Effect of elevated CO_2 on maize susceptibility to grey leaf spot disease

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Declaration

I, Kevin Daniel Scheepers declare that the dissertation, which I hereby submit for the degree Magister Scientiae, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

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In loving memory and honour of Anna-Maria (1960-2019) and Geffrey Scheepers (1960-2020)

Preface

Maize is one of the most valuable food crops around the world and global demands for the C_4 staple are predicted to double by 2050 (Leakey et al., 2004, Chaloner et al., 2021). Rising CO_2 levels will affect both natural and agricultural ecosystems in a variety of ways and potential changes to plant diseases present one of the greatest concerns for food security. Any modification in disease is dependent on the impact of CO_2 enrichment on the pathogen and host individually, as well as the specific interaction between the two constituents. Grey leaf spot (GLS) disease, which is caused by the fungal pathogen *Cercospora zeina* in Southern Africa, is one of the most damaging foliar infections of maize around the globe, but its response to the predicted future climate is currently unknown (Ward et al., 1999). Research has shown that maize physiology, morphology, molecular constitution, as well as fungal growth and pathogenicity, are all subject to change due to elevated [CO_2], however, some of the observations have been inconsistent (Leakey et al., 2006, Ainsworth and Long, 2021). The exact nature and extent of the differences is contingent on a variety of study design factors, including growth conditions, cultivar choice and the timing of measurements. Enclosed chamber trials can simulate field conditions relatively well, but chamber effects and irregular atmospheric coupling can skew results. Therefore, it is important that research into plant-pathogen interactions at elevated CO_2 levels considers the benefits and limitations of the different systems employed and assesses all results in conjunction to draw meaningful conclusions. Leaf productivity and metabolomics, as well as stomatal distribution and functionality, are all correlated to GLS disease development, based on the pathogen's mode of entry and relatively long latent period (14-21 days before symptom development) (Prins et al., 2011, Korsman et al., 2012). Consequently, their assessment offers insights into the *C. zeina*-maize interaction at ambient and elevated [CO_2], as well as detailing the physiological effect of GLS on maize leaves. Thus, this study contributes to the understanding of a prevalent maize disease in the projected climate, which allows for proactive crop management and breeding technique development.

The dissertation comprises of two chapters, a literature review and experimental chapter.

Chapter 1: Effect of elevated CO_2 on fungal diseases of C_4 plants

The first chapter is a literature review that discusses what is presently established regarding the effects of elevated CO_2 concentrations on C_4 photosynthesis, as well as maize and fungal biology. CO_2 enrichment acting in conjunction with secondary abiotic factors is also discussed, along with the research conducted on plant-pathogen interactions at elevated [CO_2].

Chapter 2: Effect of elevated CO_2 on maize susceptibility to grey leaf spot disease

The objective of the second chapter was to assess the dynamics between *C. zeina* and maize at two different CO_2 conditions in two independent trials. Maize plants were grown at current ambient (415 ppm CO_2) and elevated CO_2 levels (700 ppm) in separate growth chambers. Half of the plants in each chamber were inoculated with *C. zeina* and the remaining control plants mock-inoculated. Disease development and severity was assessed visually and molecularly via a qPCR assay, while the individual effects of CO_2 enrichment on maize and fungal growth were also measured. Gas exchange parameters and other maize leaf properties, such as the stomatal density and sugar concentrations, were quantified and compared between the different treatments.

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Summary

Atmospheric [CO_2] are projected to increase from current levels of 415 ppm to 800-1000 ppm CO_2 by 2100. Both plants and pathogens will be impacted by the altered conditions, which can have a significant effect on plant disease severity, distribution and management practices. C_4 crops, such as maize, comprise a relatively large portion of global plant primary productivity and their sustainable production is essential for maintaining food security in the impending climate. Grey leaf spot (GLS) disease, which is caused by the fungal pathogen *Cercospora zeina* in Southern Africa, is a cause for concern around the globe, but remains uncharacterised at elevated CO_2 levels. *C. zeina* is a foliar pathogen that penetrates via maize stomata and the infection efficiency is correlated with the environmental conditions. The literature indicates that plant stomata are key components in foliar diseases and can respond markedly to CO_2 enrichment by altering stomatal development. GLS causes symptomatic chlorotic lesions that can expand to full leaf blight and reduce the photosynthetic capacity of the plant. Photosynthesis can also be influenced by rising CO_2 concentrations, which can have downstream effects on leaf productivity. Therefore, the question is whether GLS disease of maize will be affected by CO_2 enrichment? The aim of this investigation was to examine the effect of elevated [CO_2] on maize susceptibility to GLS, with the null hypothesis predicting there to be no significant discrepancy in GLS disease development between ambient and elevated CO_2 treatments. B73 inbred line maize plants were grown at current ambient (415 ppm CO_2) and elevated CO_2 levels (700 ppm) in Conviron growth chambers in two separate trials. No appreciable difference in plant height was observed between the two CO_2 treatments in both trials. The photosynthetic rate (*A*) and stomatal conductance (g_{sw}) were evaluated on the inoculated and mock-inoculated maize leaves. Enhanced photosynthetic rates were detected for maize grown at elevated [CO_2] in both trials, while higher stomatal conductance was quantified on the 700 ppm CO_2-grown plants in the repeat trial only. Elevated CO_2 levels were shown to have no effect on both maize leaf stomatal density and *in vitro C. zeina* growth. Maize leaf sugar concentrations were unaffected by elevated CO_2 levels, except for greater amounts of maltose being recorded in the ambient CO_2 leaves versus their counterparts. With regards to the disease trials, *C. zeina* inoculum was applied onto the maize leaves in each chamber, while control samples were mock-inoculated. GLS disease was shown to reduce leaf photosynthetic rates and stomatal conductance, while both these parameters were positively correlated with leaf rank. There was no variance in disease development between CO_2 regimes in the first trial, according to visual scoring and a qPCR assay. Significantly greater GLS symptoms were recorded on the high CO_2-grown maize leaves for trial 2, although this is a consequence of limited disease incidence in the ambient CO_2 chamber. The Conviron environmental data along with the established literature on GLS disease

development indicate that a chamber effect occurred in trial 2. The ambient CO_2 chamber achieved a relatively lower humidity during the key period of *C. zeina* infection following inoculation (0-21 dpi), which led to reduced GLS lesion formation in comparison with the other chambers that maintained a higher relative humidity. Consequently, the conclusion is that maize susceptibility to GLS disease will not be significantly affected by elevated CO_2 concentrations, in concurrence with the trial 1 results.

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List of abbreviations

ABA	abscisic acid
ADPG-PP	ADP-glucose pyrophosphorylase
ANOVA	analysis of variance
AUDPC	area under the disease progress curve
C	carbon
$CaCO_3$	calcium carbonate
CAM	crassulacean acid metabolism
cAMP	cyclic AMP
CO_2	carbon dioxide
COR	coronatine
cpr1	cytochrome P450 reductase 1 gene
Ct	cycle threshold
CTAB	cetyltrimethylammonium bromide
dae	days after emergence
dap	days after planting
dpi	days post inoculation
dwt	dry weight
FACE	free-air CO_2 enrichment
FBPase	fructose-1,6-bisphosphatase
FOV	field of view
gDNA	genomic DNA
GLS	grey leaf spot
GO	gene ontology
gst3	glutathione *S*-transferase III gene
HCO_3^-	bicarbonate
IPCC	Intergovernmental Panel on Climate Change
JA	jasmonic acid
LC-MS	liquid chromatography-mass spectrometry
LOX	lipoxygenase
MAT	mating type
N	nitrogen
NADP-MDH	NADP-malate dehydrogenase
NADP-ME	NADP-malic enzyme
NO	nitric oxide
NPR1	non-expressor of pathogenesis-related gene 1

PAMP	pathogen-associated molecular pattern
PAR	photosynthetically active radiation
PDA	potato dextrose agar
PEPC	phosphoenolpyruvate carboxylase
PPDK	pyruvate phosphate dikinase
PPFD	photosynthetic photon flux density
ppm	parts per million
qPCR	quantitative PCR
QTL	quantitative trait loci
RH	relative humidity
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SA	salicylic acid
SD	stomatal density
Tm	melting temperature
V-stage	vegetative stage
WUE	water use efficiency

Chapter 1

Literature review: Effect of elevated CO_2 on fungal diseases of C_4 plants

1.1 Introduction

Earth is an ever-changing environment, which encompasses the adverse effects of climate change that are reflected by increases in global mean temperatures, changes in precipitation patterns and sea levels, as well as increases in greenhouse gas emissions (Meehl et al., 2007, Parry, 2019). Atmospheric CO_2 concentrations [CO_2] are increasing exponentially due to anthropogenic activities; current levels of 400 parts per million (ppm) are expected to rise to 800-1000 ppm by the end of the century (Stocker et al., 2013, IPCC, 2014). Food security will be directly impacted by future environmental conditions, which are projected to increase the incidence of plant disease outbreaks. Maize (*Zea mays* L.) is a ubiquitous C_4 crop that serves many important roles as food, fuel, and feed (Young and Long, 2000, Manderscheid et al., 2014). Maize has been studied at elevated CO_2 levels in both enclosed chamber and open field trials, which assessed a variety of physiological and molecular processes, in addition to interactions with the environment.

Elevated [CO_2] are expected to affect C_3 and C_4 plants in varying ways, with notable changes to plant biology, yield and distribution, as well as alterations on a genetic level (Drake et al., 1997, Chakraborty et al., 2000b). There is a large amount of research, which shows that C_3 plants will undoubtedly benefit from higher CO_2 levels in terms of increased primary productivity (Wand et al., 1999, Ainsworth and Long, 2005, Taub, 2010). However, the picture is not as clear with C_4 plants, as there is a relatively limited amount of research, coupled with contrasting results for this photosynthetic system (Long et al., 2006, Ainsworth and Long, 2021). In terms of the response to elevated [CO_2] in C_4 plants, selected studies have shown no significant differences in physiological parameters (Sage, 1994, Chun et al., 2011), while other analyses reported an increase in plant biomass and leaf photosynthetic rates, along with a decrease in stomatal conductance (Maroco et al., 1999, Prins et al., 2011). A third scenario represents results that indicate a significant change only when CO_2 enrichment is coupled with a secondary abiotic factor, such as drought or heat stress (Leakey et al., 2006, Kim et al., 2007, Xu et al., 2014).

Plant diseases, such as grey leaf spot (GLS) of maize, are significant threats to crop production and food sustainability. Plant-pathogen relationships will be affected by rising

[CO_2], as potential changes in either or both participants could lead to significantly different outcomes (Runion, 2003, Nazir et al., 2018). Furthermore, abiotic and biotic stresses acting in combination on plants could elicit a paradigm shift in our understanding of the disease triangle. Elevated CO_2 levels can affect host composition and resistance, as well as the pathogenicity of the microorganism (Kazan, 2018). Changes in plant physiology could have inhibitory effects on the pathogen's mode of entry or disease cycle through changes in stomatal opening or leaf composition, respectively (McElrone et al., 2005). In contrast, increased nutritional quality and reduced plant decomposition observed in CO_2-rich ecosystems, could result in greater disease severity and dissemination. Maize leaf sugars have been shown to function in cellular signalling, as well as gene regulation, and are affected by CO_2 enrichment (Gómez-Ariza et al., 2007, Bolouri Moghaddam and Van den Ende, 2012). The nutritional quality and chemistry of plant leaves could also affect foliar pathogens by altering the availability of nutrients to fuel proliferation. The pathogens themselves may also undergo physiological alterations in response to rising CO_2 concentrations that could provide or diminish a competitive advantage, including modifications to growth, metabolism and intercellular signalling (Bahn and Mühlschlegel, 2006). Bidirectional shifts between susceptibility and resistance are a major concern for the sustainability of food security and plant-derived products in the future. Understanding these changing dynamics will allow scientists and farmers to proactively plan and employ crop resistance strategies in response to climate change.

The aim of this literature review is to assess what is currently known about the effects of elevated [CO_2] on C_4 photosynthesis, and more specifically maize biology, in terms of physiological and molecular changes. The effects of secondary abiotic factors acting in addition to CO_2 enrichment, as well as the fungal response, will then be evaluated. Lastly, plant-pathogen interactions at elevated CO_2 levels will be addressed.

1.2 Comparisons of C_3 vs C_4 plant responses to elevated CO_2

Plants are capable of synthesizing carbohydrates and other vital organic biomolecules from atmospheric carbon dioxide (CO_2) in a process known as carbon fixation (Farquhar and Sharkey, 1982). Carbon fixation occurs during the light-independent reactions of photosynthesis and can occur through three different pathways, namely C_3, C_4 and crassulacean acid metabolism (CAM) photosynthesis (Ehleringer and Cerling, 2002). C_3 photosynthesis is reasoned to have evolved with the establishment of land plants and accounts for approximately 95% of the world's plant biomass. On the other hand, C_4 crop species account for 23% of global primary production, while only making up around 3% of angiosperms that employ the corresponding photosynthetic mechanism (Kellogg, 2013). The C_4 photosynthesis mechanism evolved multiple times in independent plant lineages, including 66 lineages of flowering plants and is present in many agronomically important crop species, including maize, sugarcane and sorghum (Sage et al., 2012).

C_3 photosynthesis utilises the Calvin-Benson cycle to fix atmospheric CO_2 into useable plant carbohydrates. The reaction takes place within plant mesophyll cells, with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) functioning as the chief regulatory enzyme. Rubisco is capable of utilising both CO_2 and oxygen (O_2) as substrates and its binding activity is reliant on the CO_2/O_2 ratio (Ehleringer and Cerling, 2002). The photosynthetic rate in C_3 plants is relatively efficient at ambient [CO_2], but inefficient at decreased CO_2 levels. Another shortcoming of this system is that it is particularly inefficient under high temperature or drought conditions, as these stressors cause plants to close their stomata to limit water loss and the subsequent increase in oxidative photorespiration limits the amount of CO_2 available for carbon fixation (von Caemmerer and Furbank, 2003). The C_4 photosynthesis pathway overcomes these limitations by utilising a specialized biochemical pump located within the mesophyll that creates a high intracellular CO_2 environment within the bundle sheath cell chloroplasts where the Rubisco enzyme is located. This is achieved through the activity of phosphoenolpyruvate carboxylase (PEPC), which is a highly active enzyme, but comes at the cost of creating a more energy intensive system (Ehleringer and Cerling, 2002). The ultimate result is that photosynthesis can still occur at a relatively efficient rate in low CO_2 environments or even when the plants' stomata are closed (Sage et al., 2012). However, C_4 photosynthesis is less efficient at elevated CO_2 levels than its C_3 counterparts due to the added energy usage and higher CO_2/O_2 ratio in C_3 plants. Each photosynthetic system has trade-offs and is most suitable to the environment in which they evolved.

Many studies have illustrated that elevated CO_2 levels will benefit C_3 plant species in terms of increased growth and yields (Ehleringer and Björkman, 1977, Miri et al., 2012). C_4 plants on the other hand, have shown inconsistent results when grown in CO_2-rich conditions (Table 1). Maize grown at 640 ppm CO_2 had 20% greater dry weight than plants at 330 ppm CO_2 after 30 days in well-watered soil, according to Wong (1979). A glasshouse study on maize (cv. Cromac) cultivated under water limiting conditions detected a 36% increase in total dry weight at 680 versus 340 ppm CO_2 (Morison and Gifford, 1984). In contrast, no difference in either shoot or total dry weight was seen between maize plants (cv. Summer Sweet 7200) grown with a non-limiting water supply at ambient (350-500 ppm) or double ambient [CO_2] of 650-800 ppm (Hunt et al., 1991). Maroco et al. (1999) grew maize (cv. 130 Garland Flint) at 1100 ppm CO_2 (3 times ambient) and reported a 20% increase in plant biomass in comparison to the ambient-grown plants. Driscoll et al. (2006) cultivated maize plants at 350 and 700 ppm CO_2 in controlled environment cabinets and noted that the elevated CO_2-grown plants were 23% taller than their counterparts, while there was no difference in the number of leaves, tillers or cobs between treatments (Driscoll et al., 2006). However, the researchers later reported that chamber irregularities due to deviations in cabinet specifications were responsible for the increase in growth and no change in plant height was seen in a subsequent study (Prins et al., 2011). The biomass of the C_4 grass *Paspalum dilatatum* doubled after 6 weeks at elevated [CO_2] (700 ppm), while the shoot to root ratio was reduced (Soares et al., 2008). Several free-air CO_2 enrichment (FACE) experiments have demonstrated matching aboveground maize growth rates at ambient (376-390 ppm) and elevated CO_2 levels (550-585 ppm) (Leakey et al., 2006, Manderscheid et al., 2014, Ruiz-Vera et al., 2015). The diverse plant growth conditions utilised for the assorted studies mentioned could contribute to the variable growth of C_4 plants observed, with water supply serving as a key factor. Different cultivars, chamber effects, irregular open field conditions and other artefacts can skew results, but the weight of evidence indicates that C_4 species growth will not be significantly affected by CO_2 enrichment.

The effects of elevated CO_2 levels on several other factors relating to the C_3 and C_4 photosynthetic systems have also been researched. Both C_3 and C_4 species have displayed photosynthetic acclimation to elevated [CO_2], which includes the downregulation of respiration and upregulation of pathways involved in carbon assimilation (Drake et al., 1997, Watling et al., 2000). CO_2 enrichment is thought to have a feedback inhibitory effect on Rubisco synthesis, which is supported by lower Rubisco enzyme activity and content being reported in numerous studies on C_3 species at high [CO_2], including crops such as tomato and rice (Van Oosten and Besford, 1996, Gesch et al., 1998). A significant decrease in both bundle sheath thickness and photosynthetic rates were recorded in C_4 sorghum at 700 ppm CO_2, as compared to the ambient (350 ppm CO_2) plants (Watling et al., 2000). Changes in different photosynthetic tissues and systems at elevated CO_2 levels will undoubtedly differ between C_3

and C_4 plants given their distinct adaptations, but both systems can acclimatise to changing climatic conditions.

Table 1. The effect of elevated [CO_2] on maize growth and yield.

Maize Variety	**[CO_2] ppm**	**Growth Conditions**	**Elevated [CO_2] effect**	**Reference**
Zea mays	330 vs 640	Glasshouse, well-watered, 32/20°C	Increased dry weight	**(Wong, 1979)**
Zea mays cv. Cromac	340 vs 680	Glasshouse, water limiting, 28/23°C	Increased dry weight	**(Morison and Gifford, 1984)**
Zea mays cv. Summer Sweet 7200	350-500 vs 650-800	Chamber, well-watered, 18/18°C	No effect	**(Hunt et al., 1991)**
Zea mays cv. 130 Garland Flint	350 vs 1100	Chamber, well-watered, 33/20°C	Increased biomass	**(Maroco et al., 1999)**
Zea mays cv. 34b43, Pioneer Hi-Bred International	376 vs 550	FACE[a], well-watered	No effect	**(Leakey et al., 2006)**
Zea mays cv. 34b43, Pioneer Hi-Bred International	376 vs 550	FACE, water limiting	Increased biomass	**(Leakey et al., 2006)**
Zea mays hybrid H99	350 vs 700	Chamber, well-watered, 25/19°C	No effect	**(Prins et al., 2011)**
Zea mays cv. Romario	378 vs 550	FACE, well-watered	No effect	**(Manderscheid et al., 2014)**
Zea mays cv. Romario	378 vs 550	FACE, water limiting	Increased biomass and grain yield	**(Manderscheid et al., 2014)**
Zea mays cv. 34b43, Pioneer Hi-Bred International	390 vs 585	FACE, declining precipitation, elevated temperature	No effect	**(Ruiz-Vera et al., 2015)**

[a] Free-air CO_2 enrichment.

1.3 Effect of elevated CO_2 on maize biology

1.3.1 Physiological and morphological effects

Elevated [CO_2] have been shown to have a variety of effects on maize physiology, including changes to photosynthesis, stomatal functioning and other biological processes. These modifications can lead to distinct phenotypes and have either beneficial or detrimental downstream effects on an important cereal crop (Taub, 2010). Maize has been grown under elevated [CO_2] in both enclosed chamber and FACE experiments, which have yielded contrasting results (Ainsworth and Long, 2021).

The photosynthetic rate, which can be quantified as the net CO_2 assimilation rate (*A*), is an important assessment of the productivity of a plant leaf, correlates with leaf area and is affected by high CO_2 concentrations. Maize leaf area was relatively unchanged between plants grown in a glasshouse at 330 or 640 ppm CO_2, while the photosynthetic rate was enhanced (1.2X) by the elevated CO_2 levels in well-fertilised plants (Wong, 1979). Maroco et al. (1999) studied maize at 350 and 1100 ppm CO_2 and reported a 23% increase in leaf area at elevated [CO_2] under high light conditions, which corresponded to a 15% increase in the light-saturated rate of photosynthesis. Conversely, no significant differences in maize leaf area, photosynthesis or the activity of vital photosynthetic enzymes were observed by Leakey et al. (2006) in plants cultivated in the open field (FACE) at 550 ppm CO_2 when compared to the ambient (376 ppm CO_2) plants. Both Driscoll et al. (2006) and Prins et al. (2011) observed no significant change in maize photosynthesis between ambient (350 ppm CO_2) and elevated CO_2 (700 ppm) treatments in chamber trials. Similarly, maize photosynthetic rates were not affected by twice-ambient [CO_2] (800 ppm) in a FACE experiment (Vaughan et al., 2014). CO_2 enrichment has shown variable responses with regards to maize photosynthesis, which suggests that changes in physiology could occur at specific stages or in a leaf rank-dependent manner.

Plant stomata provide a key pathway for gas exchange between the leaf and surrounding atmosphere. Changes in stomatal opening, size and number, along with the movement of gasses through these leaf pores have been documented in CO_2 enrichment studies (Xu et al., 2016). CO_2 is known to function in signal transduction processes, which affect leaf development and structure. Plants can thus control stomatal numbers by transmitting information regarding [CO_2] from older to younger leaves, with carbonic anhydrases playing a key role in this process (Lake et al., 2001, Hu et al., 2010). Maize leaves are amphistomatous in that they have stomata on both the adaxial and abaxial surfaces, though they are more abundant on the abaxial side (Juarez et al., 2004). In a chamber study, Maroco et al. (1999)

reported a 71% decrease in stomatal conductance, which directly corresponds to greater stomatal closure, in maize plants at 1100 vs 350 ppm CO_2. Greater stomatal densities (20-30%) on both sides of the leaf were observed in mature leaves, as compared to young leaves at ambient and elevated [CO_2]. It was also noted that the 1100 ppm CO_2-grown plants had 6-16% lower stomatal densities than their ambient counterparts on both mature and young leaves (Maroco et al., 1999). In a FACE study by McElrone et al. (2005), a 21-36% decrease in stomatal conductance was reported, while stomatal density and size were unaffected by the high CO_2 levels (550 ppm). In contrast, Driscoll et al. (2006) documented an increase in stomatal size at elevated [CO_2] (700 ppm) in maize, but also noted that the number of stomata remained unchanged between the ambient and elevated CO_2 treatments. In a follow-up study, lower stomatal conductance was detected in high CO_2-grown (700 ppm) maize leaves in comparison to the ambient (350 ppm CO_2) plants and this effect was more pronounced in the younger leaves (Prins et al., 2011). Maize stomata are an important channel for gas exchange and their response to CO_2 enrichment via increased closure will have many downstream effects with regards to the plant’s interaction with the surrounding environment. However, the exact mechanism and timing of stomatal control remains to be elucidated.

Other physiological processes, such as water use, respiration and transpiration, are also affected by elevated CO_2 levels, highlighting the complex interplay required to maintain homeostasis in fluctuating conditions. Water use efficiency (WUE) is the proportion of water utilised in plant metabolism to water lost through transpiration (Baldocchi, 1994). A twofold increase in WUE was reported in maize at high CO_2 concentrations (640 ppm), which was attributed to greater photosynthesis in some samples and decreased transpiration in other plants (Wong, 1979). Maize plants grown at 1100 ppm CO_2 had a 225% rise in WUE, as well as a 100% higher dark respiration rate, as compared to the plants in ambient conditions. Decreased carboxylation efficiencies of 23% were also observed when recorded under limiting [CO_2] (Maroco et al., 1999). Maize plant photorespiration and leaf C/N ratios were unaffected by the CO_2 concentration (350 vs 700 ppm) in one chamber study. The transpiration rates declined in lower leaf ranks, but not upper ranks at high [CO_2], while the water use efficiency of both ranks increased (Prins et al., 2011). Many plant processes are linked, and individual changes can have a knock-on effect that determines the cellular response to altered atmospheric conditions. Maize management in terms of irrigation patterns and growing season selection will need to be re-evaluated to account for varying crop physiology.

Maize morphology has been documented to be influenced by high CO_2 levels, with leaf and root development and structure the most affected plant systems. Maroco et al. (1999) analysed leaf cross-sections of maize cultivated in ambient and elevated [CO_2] and found that there was no difference in the size of both bundle sheath and mesophyll cells. Additionally, no change in the localisation of Rubisco and PEPC was observed between CO_2 treatments, with the two enzymes confined to the bundle sheath chloroplasts and mesophyll cytoplasm, respectively. Driscoll et al. (2006) found that there were larger epidermal cells on both the top and bottom surfaces of maize leaves for plants grown at 700 versus 350 ppm CO_2. Han et al. (2020) studied maize root development at 0, 1000 and 2000 ppm CO_2 via gas injections into the soil and noted that the high CO_2 levels inhibited root growth. There was a 7.7% decrease in mean root length at 1000 ppm CO_2 and 44.7% decrease at 2000 ppm CO_2, which was attributed to changes to fine and not coarse roots (Han et al., 2020). The literature establishes that changes in leaf tissue development and root structure at elevated [CO_2] will affect leaf composition and maize morphology.

1.3.2 Molecular and genetic effects

Elevated [CO_2] have been shown to elicit noteworthy changes to maize gene expression, as well as the transcriptome, proteome and metabolome. These multi-level intercellular effects can elicit deviations in the production of certain compounds, metabolites or enzymes (Kim et al., 2006, Ge et al., 2018). There have also been studies demonstrating altered plant chemistry, which leads to changes in the nutrient composition and quality of maize. Many biomolecules and metabolites play multiple roles within plants and can involve complex feedback mechanisms governing regulation and a range of downstream responses (Sicher and Barnaby, 2012).

The transcriptome offers valuable insights into the differential expression of genes in the diverse environments that plants grow and presents a key vantage point to observe cellular changes in maize in the future climate. Microarray analysis revealed an unchanged leaf transcriptome profile in response to CO_2 enrichment (700 ppm) in maize plants (Prins et al., 2011). In contrast, Ge et al. (2018) examined the effect of elevated [CO_2] on the maize leaf transcriptome by growing plants at ten times ambient levels of 4000 ppm CO_2 for 7 and 14 days and observed that over 1300 genes were either up- or downregulated significantly between treatments. Gene functional annotation revealed gene ontology (GO) enrichment in the areas of protein phosphorylation and ubiquitination, oxidation-reduction, plant organ development, MAPK signalling and transcription factors functioning in plant defence (Ge et al., 2018). High [CO_2]-induced changes to the transcriptome, such as those reported in the literature, suggest that altered expression levels can occur in genes related to CO_2 signalling and post-translational modifications. However, it is also possible that sufficient time is not available for significant changes to be observed in one growing season or that there could be a CO_2 threshold effect.

Proteins and enzymes form the building blocks and functional components of plant cells and their production, functionality and activity can be affected by elevated [CO_2]. Maroco et al. (1999) reported a 22% decrease in leaf protein content in maize plants grown in CO_2-rich conditions (1100 ppm). Both lower Rubisco content and activity (8-9%) was observed in the leaves of elevated CO_2-grown plants at 8 and 30 days after emergence (dae). The phosphoenolpyruvate carboxylase (PEPC) enzyme, which functions in creating a high CO_2 environment around Rubisco in C_4 plants, showed decreased activity at 1100 ppm CO_2 at both 8 (23%) and 30 dae (5%) (Maroco et al., 1999). The activity of NADP-malate dehydrogenase (NADP-MDH), a C_4 cycle enzyme, decreased by 31% at elevated [CO_2] on a leaf-area basis at 30 dae. On the other hand, some FACE studies have shown that neither total leaf protein, free amino acids nor leaf N are altered by elevated CO_2 levels (Leakey et al., 2006). Prins et al. (2011) showed that several proteins differed in abundance between ambient and elevated CO_2 treatments. The majority of proteins could not be identified using liquid chromatography-mass spectrometry (LC-MS/MS), but those that could were associated with photosynthesis, respiration and protein metabolism (Prins et al., 2011). These changes in the activity of key C_3 and C_4 enzymes highlight the adaptability of the photosynthesis system and provide an indication that maize plants will be able to acclimate to future growing conditions through proteome modifications.

Changes to leaf composition, nutrient content and the metabolome at elevated CO_2 levels can affect plant ecosystems and interactions, in addition to the nutritional value of food crops. Sugars, such as sucrose and glucose, have been demonstrated to play roles in both the repression and stimulation of gene expression in maize, which allows for feedback regulation of photosynthesis in the plant (Sheen, 1994). Fructose 1,6-bisphosphatase (FBPase) (8%) and ADP-glucose pyrophosphorylase (ADPG-PP) (36%), which are two enzymes required in starch and sucrose biosynthesis, showed greater activity in maize leaves at high [CO_2] than in their ambient CO_2 grown counterparts (Maroco et al., 1999). Genes encoding PEPC, pyruvate phosphate dikinase (PPDK) and Rubisco's small subunit are all downregulated by glucose, while ADP-glucose pyrophosphorylase (ADPG-PP) and sucrose synthase genes are upregulated (Van Oosten and Besford, 1996, Koch, 1996). Leakey et al. (2006) observed no significant difference in either total leaf carbohydrate content or the constituent sugars, including glucose, sucrose, fructose and starch, between the ambient (376 ppm CO_2) and elevated (550 ppm CO_2) treatments. In a 2011 maize trial, elevated [CO_2] were shown to significantly increase the amount of leaf sugars, such as glucose, galactose and mannose, as well as linoleic and linolenic fatty acids. On the other hand, myo-inositol and hydroxybenzoic acid, which function in salicylic acid (SA) metabolism, along with proline, glutarate and protein carbonyl groups, all decreased due to CO_2 enrichment, while photorespiratory metabolites and leaf N status were not affected (Prins et al., 2011). These high CO_2-induced changes in enzymes associated with sugar production suggest that the dual role sugars play in maize regulation and nutrient content is subject to modification in the future climate.

Plant sugars and hormones play a variety of roles in signalling and regulating cellular responses and research has demonstrated that they are influenced by elevated [CO_2]. Carbohydrates and phytohormones function in co-regulating plant defences and elevated quantities of sugars can induce host resistance during pathogen infection by providing the additional energy required for downstream reactions (Gómez-Ariza et al., 2007, Bolouri Moghaddam and Van den Ende, 2012). CO_2 enrichment has been shown to affect plant defence hormones, including the stimulation of SA production and suppression of jasmonic acid (JA) and lipoxygenase (LOX) synthesis (Casteel et al., 2012, Vaughan et al., 2014). A regulatory protein, non-expressor of pathogenesis-related genes 1 (NPR1), controls the interaction between SA and JA, in which the former downregulates the latter (Spoel et al., 2003). High CO_2 levels are theorised to affect the plant redox state, which subsequently activates NPR1 (Spoel and Loake, 2011). Phytohormones, such as abscisic acid (ABA), JA, brassinosteroids, cytokinins and ethylene, as well as reactive oxygen species (ROS), all function in regulating leaf stomatal responses, which is an important component of plant defensive strategies (Kazan, 2018). It is evident from the literature that there is a complex interplay within maize plants and any individual CO_2 effect on plant metabolites can have many cascading downstream effects.

1.3.3 Interactive effects with secondary abiotic stress factors

Environmental changes do not occur in isolation; therefore, it is prudent to consider abiotic stresses acting in conjunction. In terms of the response to elevated CO_2 concentrations in C_4 plants, while some researchers have reported a change in biomass and gas exchange parameters (Maroco et al., 1999, Driscoll et al., 2006), other studies have shown no significant differences (Sage, 1994). A third scenario represents results that indicate a change to growth and photosynthetic rates only when CO_2 enrichment is coupled with a secondary abiotic factor (Leakey et al., 2006, Kim et al., 2007). Researchers have conducted studies evaluating elevated [CO_2] in addition to heat stress, drought, and other greenhouse gases or chemicals.

Another consequence of climate change is altered rainfall patterns, which can cause unpredictable droughts and modify soil water content. Leakey et al. (2006) conducted a FACE experiment in which they examined maize grown at 376 and 550 ppm CO_2, with water stress as an additional independent variable. Measurements were taken at multiple times throughout the day and at five different stages of maize development and the researchers noted that there was no significant change to biomass, yield and photosynthetic rates in the absence of water stress. The *in vitro* and *in vivo* activity of key photosynthetic enzymes, such as PEPC, PPDK and Rubisco, in addition to metabolic markers of C and N status, also remained unaffected by the CO_2 concentration (Leakey et al., 2006). Reduced maize water use was reflected by 31% greater soil moisture and a 34% reduction in stomatal conductance at elevated [CO_2] (550 ppm) versus ambient. The leaves grown at 550 ppm CO_2 showed significantly lower transpiration rates than those at 376 ppm CO_2, but leaf water status was unaffected (Leakey et al., 2006). During drought conditions however, the diminished water use caused by elevated CO_2 levels can result in the stimulation of photosynthesis and increased yield (Ghannoum et al., 2000, Leakey et al., 2004). Markelz et al. (2011) studied the interactive effects of elevated [CO_2] and drought on maize in a FACE trial and reported 44% lower photosynthetic rates and 57% lower stomatal conductance in the high CO_2-grown plants under drought stress compared to the current ambient counterparts. The *in vivo* activity of PEPC was decreased by 58%, but this effect, as well as the reduced leaf photosynthetic capacity, was not detected under well-watered conditions, highlighting the restorative effect of CO_2 enrichment (Markelz et al., 2011). Another maize FACE study showed that elevated CO_2 (550 ppm) treatment resulted in a 24% increase in total biomass and 41% greater grain yield during drought stress only (Manderscheid et al., 2014). These results indicate that the CO_2 levels predicted for the future can have an ameliorating effect on water-stressed maize and thus bolster yields.

Rising CO_2 levels are predicted to be accompanied by increasing global mean temperatures, with a 1.5°C increase anticipated by the end of the century (IPCC, 2018). It has been shown that the optimal temperature for photosynthesis in maize leaves is 34°C at both ambient and elevated [CO_2] (Kim et al., 2007, Hatfield et al., 2011). Kim et al. (2007) grew maize in sunlit growth chambers at ambient (370 ppm CO_2) and double ambient CO_2 (750 ppm) at five different day/night temperatures (19/13, 25/19, 31/25, 35/29 and 38.5/32.5°C). It was reported that there is no high CO_2 effect on leaf area, photosynthesis, biomass and PPDK activity. Although, the *in vitro* activity of other C_4 enzymes, namely PEPC and NADP-malic enzyme (NADP-ME), as well as the carboxylation efficiency, all decreased significantly due to CO_2 enrichment. High temperatures were shown to reduce leaf area and biomass, although none of the parameters measured at these levels differed between CO_2 regimes (Kim et al., 2007). On the other hand, Ruiz-Vera et al. (2015) examined the effect of elevated CO_2 (585 ppm) and temperatures (2.7°C above ambient) on maize in a FACE trial and found that there was no interactive effect on photosynthesis. Furthermore, the researchers reported that elevated CO_2 concentrations do not have a significant effect on either yield or photosynthetic rates but do reduce stomatal conductance by 33%. Decreased seed yield and reduced photosynthesis were also observed at higher temperatures during the latter half of the season, while aboveground biomass was not affected by either treatment (Ruiz-Vera et al., 2015). There is a known correlation between photosynthetic rates and leaf N content, which decreased in older maize plants (Ghannoum and Conroy, 1998, Ruiz-Vera et al., 2015). It is therefore apparent that the combination of elevated [CO_2] and temperatures can have a synergistic or antagonistic effect, depending on the particular maize system.

Biotic and abiotic stresses acting in conjunction presents a major threat for all crops, however, the interactive effects of CO_2 enrichment, drought and heat stress are not well understood on maize (Vaughan et al., 2018). Xu et al. (2014) examined the combined effects of elevated CO_2, warming and precipitation on the C_4 grass, *Cleistogenes squarrosa*, and reported that the relationship between elevated CO_2 and rising temperature is dependent on water status and is variable among species. The mitigating effect of high CO_2 on combined drought and heat stress is contingent on the dominant stressor (usually drought), as each stress causes different physiological changes that require the corresponding plant adaptive mechanism (Abdelhakim et al., 2022). Therefore, the interplay between maize transpiration rates, WUE, photosynthetic rates and stomatal control will determine the outcome of the multifactorial effects of climate change on maize biology and productivity.

1.4 Effect of elevated CO_2 on fungal biology

In fungi, CO_2 influences essential biological characteristics, such as growth, morphology, metabolism, spore production and pathogenicity. Additionally, CO_2 functions as a signalling molecule, which can be transported across membranes by diffusion. Carbonic anhydrase and adenylyl cyclase are two fungal enzymes that play important roles in CO_2 perception (Bahn and Mühlschlegel, 2006). Carbonic anhydrase catalyses CO_2/bicarbonate (HCO_3^-) homeostasis, whereas adenylyl cyclase synthesizes cyclic AMP (cAMP), which functions as a second messenger that relays extracellular signals to intracellular effectors (Parrent and Vilgalys, 2007, Pohlers et al., 2017). CO_2 is sensed via two pathways in fungi, in the first, direct activation of adenylyl cyclase regulates virulence, as observed in pathogenic yeast (Rocha et al., 2001, Alspaugh et al., 2002). The second pathway, which is independent of adenylyl cyclase, plays a vital role in fungal metabolism, but remains poorly characterised (Pohlers et al., 2017).

A small number of CO_2 enrichment studies have analysed the growth and development of fungal pathogens *in vitro* (Table 2). In one *in vitro* study, Bécard and Piché (1989) reported that *Gigaspora margarita* hyphal growth was significantly greater at 0.5% than 0.03% CO_2 on common medium. In contrast, increasing CO_2 enrichment (10-25% CO_2) resulted in decreased *in vitro* growth of the brown rot fungus *Monilinia fructicola* on potato dextrose agar (PDA), with complete inhibition of mycelial growth occurring at 30% [CO_2] (Tian et al., 2001). *Phytophthora parasitica* (oomycete) was cultured on V8C agar at 350 and 700 ppm CO_2 and no difference in hyphal growth was observed between treatments *in vitro* (Jwa and Walling, 2001). McElrone et al. (2005) axenically cultured the fungal foliar pathogen *Phyllosticta minima* on PDA at ambient (360 ppm CO_2) and elevated [CO_2] (560 ppm). The chamber experiment showed that the *in vitro* growth rate of *P. minima* was enhanced by 17% under elevated CO_2 levels (McElrone et al., 2005). The *in vitro* colony growth of *Aspergillus flavus* reduced in response to rising CO_2 concentrations (25-75%) on PDA (Giorni et al., 2008). Therefore, variable outcomes should be anticipated for the range of fungal pathogens, although, a high CO_2 environment can be demonstrably influential on microbes in a host-independent manner.

The *in planta* growth and pathogenicity of several fungal pathogens have been evaluated at high [CO_2] (Table 2). Hibberd et al. (1996b) reported that *Erysiphe graminis* colonies were larger and had significantly faster growth rates *in planta* at elevated CO_2 levels (700 ppm) versus ambient (350 ppm CO_2), which was hypothesized to be due to a greater availability of host carbohydrates. The biotrophic fungus, which causes powdery mildew, was shown to have reduced conidial development that restricted leaf penetration during infection at high [CO_2], but this was due to host metabolism rather than a direct CO_2 effect (Hibberd et al., 1996b). Germ tube development on the other hand, was unaffected by the CO_2 concentration (Hibberd et al., 1996b). Conidial germination, germ tube growth and appressoria production were all delayed or decreased in response to CO_2 enrichment (700 ppm) in *Colletotrichum gloeosporioides*, a necrotrophic fungal pathogen that causes anthracnose (Chakraborty et al., 2000a). The researchers also reported significantly greater spore production and a longer incubation period at 700 versus 350 ppm CO_2, while the latent period was unaffected. However, an extended latent period was detected in the rubber vine rust fungus *Maravalia cryptostegiae* at high [CO_2], along with an increase in spore production (Coakley et al., 1999). A significant increase in *C. gloeosporioides* aggressiveness and fecundity was documented over multiple infection cycles at elevated CO_2 levels (Chakraborty and Datta, 2003). *Fusarium verticillioides* biomass increased significantly in stalk-inoculated maize at twice-ambient [CO_2] (800 ppm), although production levels of the mycotoxin fumonisin were not altered (Vaughan et al., 2014). On the other hand, *in planta* growth of two different subspecies of the hemibiotrophic pathogen, *Fusarium oxysporum*, was unaffected by elevated CO_2 concentrations of 800 ppm (Ferrocino et al., 2013, Chitarra et al., 2015). These results highlight the variation in fungal responses to high CO_2 concentrations and that changes to pathogen growth, fitness and the infection cycle are likely a consequence of climate change.

The effect of elevated [CO_2] on fungal plasticity and lifestyle has been examined in a few studies. CO_2 levels has been shown to have a substantial effect on *Candida albicans* and *Cryptococcus neoforman*s differentiation and growth (Bahn and Mühlschlegel, 2006). The two pathogenic fungi are able to adjust to a broad range of CO_2 concentrations based on the diverse environmental climates encountered during their life cycles (Frame et al., 1972, Hall et al., 2010). Fungal virulence factors are known to be affected by high CO_2 levels, which promote filamentation in *C. albicans* and capsule synthesis in *C. neoforman*s (Granger et al., 1985, Rocha et al., 2001, Alspaugh et al., 2002). Sheth et al. (2005) observed that elevated [CO_2] caused *C. albicans* to invade the underlying chocolate agar or induce filamentation, while non-pathogenic fungi showed no response. The results indicate that some fungi can adjust to drastically different [CO_2] through inter- and intracellular modifications.

Table 2. The effect of elevated [CO_2] on fungal growth and development.

[CO_2]	**Fungus**	**Disease**	**Elevated [CO_2] effect**	**Reference**
		***In vitro* studies**		
5% (50000 ppm)	*Cryptococcus neoformans* (commensal)	Cryptococcosis (humans)	Enhanced capsule synthesis	**(Granger et al., 1985)**
0.03 vs 0.5% (300 vs 5000 ppm)	*Gigaspora margarita* (biotroph)	N/A[a]	Enhanced hyphal growth	**(Bécard and Piché, 1989)**
0 vs 30% (300000 ppm)	*Monilinia fructicola* (necrotroph)	Brown rot	Inhibited mycelial growth	**(Tian et al., 2001)**
350 vs 700 ppm	*Phytophthora parasitica** (hemibiotroph)	Root rot	No effect	**(Jwa and Walling, 2001)**
5% (50000 ppm)	*Candida albicans* (commensal)	Candidiasis (humans)	Enhanced filamentation	**(Rocha et al., 2001)**
360 vs 560 ppm	*Phyllosticta minima*	Foliar leaf spot	Enhanced growth rate	**(McElrone et al., 2005)**
6% (60000 ppm)	*Candida albicans* (commensal)	Candidiasis (humans)	Enhanced filamentation	**(Sheth et al., 2005)**
0 vs 75% (750000 ppm)	*Aspergillus flavus* (saprophyte)	Ear rot	Reduced colony growth	**(Giorni et al., 2008)**
		***In planta* studies**		
350 vs 700 ppm	*Erysiphe graminis* (biotroph)	Powdery mildew	Larger colonies, enhanced growth rate, reduced conidial development	**(Hibberd et al., 1996b)**
350 vs 700 ppm	*Maravalia cryptostegiae* (biotroph)	Rust	Extended latent period, enhanced spore production	**(Coakley et al., 1999)**
350 vs 700 ppm	*Colletotrichum gloeosporioides* (necrotroph)	Anthracnose	Reduced conidial germination, germ tube growth and appressoria production, enhanced spore production, extended incubation period	**(Chakraborty et al., 2000a)**

350 vs 700 ppm (25 infection cycles)	*Colletotrichum gloeosporioides* (necrotroph)	Anthracnose	Enhanced aggressiveness and fecundity	**(Chakraborty and Datta, 2003)**
400 vs 800 ppm	*Fusarium oxysporum* (hemibiotroph)	Fusarium wilt	No effect	**(Ferrocino et al., 2013)**
400 vs 800 ppm	*Fusarium verticillioides* (endophyte)	Stalk and ear rot	Enhanced biomass	**(Vaughan et al., 2014)**
400 vs 800 ppm	*Fusarium oxysporum* (hemibiotroph)	Fusarium wilt	No effect	**(Chitarra et al., 2015)**

* Oomycete. [a] Not applicable.

1.5 Effect of elevated CO_2 on plant-pathogen interactions

Plant growth is affected by numerous diseases caused by a variety of microorganisms, which reduce yields and primary productivity. However, there has been limited research on the effect of CO_2 enrichment on plant diseases (Garrett et al., 2006). Plant-pathogen interactions at elevated [CO_2] are difficult to predict and dependent on several factors, including the plant and pathogen type and strain, the plant pathosystem, as well as the physiological and molecular effects of elevated CO_2 levels on each individual organism. The outcomes of these compounding effects could drive disease development in either direction, depending on the extent and correlation of the stimuli (Boonekamp, 2012). The pertinent gap in the literature is that many of the most devastating diseases of important crops, such as grey leaf spot (GLS) of maize, have not yet been evaluated in CO_2-rich environments. Furthermore, pathogens are predicted to adapt quicker to range shifts, as compared to plants (Coakley, 1995). The clear concern is increased incidence and severity of plant diseases in the projected future climate, which could threaten food security, in addition to many other vital industries.

Research on plant-pathogen interactions under elevated [CO_2] has produced variable results on both C_3 and C_4 species (Table 3), with cases of increased, decreased and unchanged susceptibility and severity being reported (Garrett et al., 2006). Selected CO_2 enrichment studies have documented incidences of increased susceptibility to leaf blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) in C_3 rice (Kobayashi et al., 2006) and greater severity of fungal infection in the C_4 grass *Spartina patens* (Thompson and Drake, 1994). C_3 species, which are expected to have increased growth rates, biomass and nutritional quality at elevated CO_2 levels, could consequently have denser canopies that increases humidity to the benefit of pathogens (Debela and Tola, 2018). Humid conditions would be particularly beneficial to foliar diseases, including leaf spots, powdery mildews, blights and rusts (Coakley et al., 1999). Many pathogens overwinter on plant litter, which provides inoculum for the following growing season. CO_2 enrichment (600-700 ppm) has been shown to increase the C:N ratio of C_3 plant litter, while this litter decomposes at a significantly reduced rate. However, no change in litter decomposition or quality at elevated [CO_2] was observed in C_4 plants (Ball, 1997). Sharma et al. (2015) studied *Phytophthora cajani* (oomycete) infection of pigeon pea plants (C_3) in a chamber study and found that Phytophthora blight incidence was identical between 380 ppm, 550 ppm and 700 ppm CO_2 treatments. Ferrocino et al. (2013) conducted a phytotron trial and documented that elevated CO_2 levels (800 ppm) did not have a significant effect on Fusarium wilt (*F. oxysporum* f.sp. *lactucae*) severity of C_3 lettuce. In contrast, Chitarra et al. (2015) examined Fusarium wilt (*F. oxysporum* f.sp. *conglutinans*) on rocket, which is a C_3-C_4 intermediate, and detected an increase in disease incidence due to CO_2 enrichment (800 ppm). Tomato plants (C_3) grown in CO_2-rich conditions (700-800 ppm) showed enhanced tolerance to buckeye rot (*Phytophthora parasitica*), the *tobacco mosaic virus* (TMV) and bacterial speck disease (*Pseudomonas syringae*) (Jwa and Walling, 2001, Zhang et al., 2015). In contrast, grey mould caused by *Botrytis cinerea* produced more severe disease symptoms (Zhang et al., 2015). Vaughan et al. (2014) reported that elevated CO_2 levels (800 ppm) resulted in increased maize susceptibility to *F. verticillioides*, as compared to ambient (400 ppm CO_2), while mycotoxin (fumonisin) content was unaffected. Maize defences in the form of LOXs, JA and phytoalexins were attenuated by elevated CO_2 concentrations, which corresponded to lowered resistance (Vaughan et al., 2014). Blackleg disease of canola (C_3), which is caused by the fungus *Leptosphaeria maculans*, displayed lower disease severity at 800 ppm CO_2 than ambient (400-600 ppm CO_2) (Zou et al., 2019). There does not seem to be a clear distinction between C_3 and C_4 plants with regards to disease development at elevated [CO_2], which is consistent with the variation observed within and between each system.

Many foliar diseases are caused by pathogens entering through the leaf stomata, hence plants have developed the ability to close their stomata to restrict entry (Melotto et al., 2017). The microclimatic environment on the leaf surface could also be affected by stomatal closure, as it can decrease localized humidity around stomata. Stomatal closure can be induced by pathogen-associated molecular patterns (PAMPs), including fungal toxins and cell wall components (Lake and Wade, 2009). Plant stomatal responses can be manipulated by pathogens to overcome the host's defences, *Pseudomonas syringae* for example, utilises coronatine (COR) to reopen tomato leaf stomata and allow bacterial entry. COR is a toxin and analogue of JA, which is a plant hormone that functions in signalling and host defence (Lake et al., 2002). Elevated CO_2 levels are known to reduce stomatal conductance and thus stomatal openings, which would affect foliar pathogens that enter through these apertures. However, Thompson et al. (1993) reported that the severity of powdery mildew (*Erysiphe graminis*) infection on winter wheat (C_3) was unaffected by elevated [CO_2] (700 ppm). Hibberd et al. (1996b) studied the relationship between barley (C_3) and *E. graminis* at elevated CO_2 levels (700 ppm) in a chamber trial and documented a significant decrease in disease development. *E. graminis* is a biotrophic pathogen and was shown to have truncated primary penetration following inoculation of barley leaves at 700 ppm CO_2, as compared to ambient [CO_2]. The high CO_2-grown leaves had increased carbohydrate status, which could provide more energy for plant resistance strategies (Hibberd et al., 1996a, Hibberd et al., 1996b). Elevated CO_2 levels were shown to increase aggressiveness of the biotrophic fungal pathogen *Erysiphe cichoracearum*, which is another one of the causal agents of powdery mildew. Host Arabidopsis plants (C_3) displayed greater stomatal densities and guard cell lengths due to CO_2 enrichment (800 ppm), which is linked to increased pathogen entry (Lake and Wade, 2009). Li et al. (2015) showed that both stomatal aperture and *P. syringae* infection of tomato plants were significantly reduced at 800 ppm CO_2, as compared to the ambient (380 ppm CO_2) plants. This defence was associated with nitric oxide (NO), whose production in the stomatal guard cells was also increased in response to CO_2 enrichment. However, stomata-independent pathways also exist to thwart *P. syringae* infection, as syringe-inoculated plants were still resistant at high [CO_2] despite stomatal defences being circumvented (Li et al., 2015). The findings discussed suggest that variation in pathogenicity in CO_2-rich environments is host specific and associated with stomatal responses for foliar diseases.

Disease development has been assessed at elevated [CO_2] in both FACE and enclosed chamber studies. Chakraborty et al. (2000a) conducted both enclosed chamber and open field trials on anthracnose on *Stylosanthes scabra* plants (C_3) at elevated CO_2 concentrations (700 ppm). It was found that the high CO_2-grown plants were relatively taller, had increased biomass and more leaves than their ambient counterparts in both trials. For the chamber study, reduced severity of anthracnose was observed in susceptible legume plants at elevated CO_2 levels, whereas there was no change in a partially resistant cultivar. *C. gloeosporioides* primarily enters through leaf stomata, which reduce in aperture at high [CO_2] and may limit pathogen penetration (Coakley et al., 1999, Chakraborty et al., 2000a). On the other hand, an increase in disease severity at elevated [CO_2] was seen in the open field study, though it was not a significant change. These results indicate that the microclimate in the field, which is difficult to replicate in chambers, contributes to anthracnose development (Chakraborty et al., 2000a). In a FACE trial by McElrone et al. (2005), elevated CO_2 levels (200 ppm above ambient) were reported to reduce the incidence of foliar leaf spot disease of *Acer rubrum* (C_3) by as much as 27%. Decreased disease severity (up to 50%) was observed at high [CO_2] in the *P. minima*-infected red maple plants. There was also a 21-36% reduction in stomatal conductance that could restrict fungal entry, which is relevant as scanning electron microscopy showed that *P. minima* enters through the stomata using conidia germ tubes (McElrone et al., 2005). However, the researchers posited that the decrease in disease observed in the elevated CO_2-grown plants may be due to modified leaf chemistry and diminished nutritive quality. This was reflected by lower N content (20%) and increased production (14-15%) of defence-related compounds, i.e. tannins and phenolics (McElrone et al., 2005). A difference in disease outcomes between FACE and chamber trials demonstrates the influence of the environment on plant-pathogen interactions.

Host and pathogen evolution in response to CO_2 enrichment further complicates plant-pathogen interactions, with the shorter life cycles of microorganisms favouring their adaptation (Coakley et al., 1999). More disease-conducive microclimates, improved fecundity and additional pathogen generations per growing season could all lower host resistance, while enhanced plant defence through physiological changes could have the opposite effect. Polycyclic disease development of anthracnose was assessed at ambient (350 ppm CO_2) and elevated [CO_2] (700 ppm) over 25 consecutive infection cycles in a chamber study. Escalating disease severity and rising *C. gloeosporioides* fecundity were observed over time in the high CO_2 chamber, as compared to ambient (Chakraborty and Datta, 2003). A CO_2-rich climate could also affect the distribution of host and pathogen species, as a consequence the hotspots for particular diseases may shift into new geographic locations or infect different hosts (Coakley, 1995, Coakley et al., 1999).

Table 3. The effect of elevated [CO_2] on plant-pathogen interactions.

Trial Design	**Plant**	**Pathogen**	**Disease**	**Elevated [CO_2] effect**	**Reference**
		C_4 species			
Open-top chamber – 350 vs 700 ppm [CO_2]	*Spartina patens* – grass	Unknown fungus	Stem and leaf lesions	Increased severity	**(Thompson and Drake, 1994)**
Chamber – 400 vs 800 ppm [CO_2]	*Zea mays* – maize	*Fusarium verticillioides* (fungus)	Stalk and ear rot	Increased severity	**(Vaughan et al., 2014)**
Phytotron – 400 vs 800 ppm [CO_2]	*Eruca sativa* – rocket (C_3-C_4 intermediate)	*Fusarium oxysporum* f.sp. *conglutinans* (fungus)	Fusarium wilt	Increased severity	**(Chitarra et al., 2015)**
		C_3 species			
Glasshouse – 350 vs 700 ppm [CO_2]	*Triticum aestivum* – winter wheat	*Erysiphe graminis* (fungus)	Powdery mildew	No effect	**(Thompson et al., 1993)**
Chamber – 350 vs 700 ppm [CO_2]	*Hordeum vulgare* – barley	*Erysiphe graminis* (fungus)	Powdery mildew	Decreased severity	**(Hibberd et al., 1996b)**
Chamber – 350 vs 700 ppm [CO_2]	*Lycopersicon esculentum (tomato)*	*Phytophthora parasitica* (oomycete)	Root rot	Decreased severity	**(Jwa and Walling, 2001)**
Chamber – 350 vs 700 ppm [CO_2]	*Stylosanthes scabra* – shrubby stylo	*Colletotrichum gloeosporioides* (fungus)	Anthracnose	Decreased severity	**(Chakraborty et al., 2000a)**
Chamber – 350 vs 700 ppm [CO_2] (25 infection cycles)	*Stylosanthes scabra* – shrubby stylo	*Colletotrichum gloeosporioides* (fungus)	Anthracnose	Increased severity	**(Chakraborty and Datta, 2003)**
Open field – 350 vs 550 ppm [CO_2]	*Acer rubrum* – red maple	*Phyllosticta minima* (fungus)	Foliar leaf spot	Decreased severity	**(McElrone et al., 2005)**
FACE^a – 370 vs 620 ppm [CO_2]	*Oryza sativa* – rice	*Magnaporthe oryzae* (fungus)	Leaf blast	Increased severity	**(Kobayashi et al., 2006)**
FACE – 370 vs 620 ppm [CO_2]	*Oryza sativa* – rice	*Rhizoctonia solani* (fungus)	Sheath blight	Increased severity	**(Kobayashi et al., 2006)**
Chamber – 400 vs 800 ppm [CO_2]	*Arabidopsis thaliana* – Arabidopsis	*Erysiphe cichoracearum* (fungus)	Powdery mildew	Increased severity	**(Lake and Wade, 2009)**

Phytotron – 400 vs 800 ppm $[CO_2]$	*Lactuca sativa* – lettuce	*Fusarium oxysporum* f.sp. *lactucae* (fungus)	Fusarium wilt	No effect	**(Ferrocino et al., 2013)**
Chamber – 380 vs 550 vs 700 ppm $[CO_2]$	*Cajanus cajan* – pigeonpea	*Phytophthora cajani* (oomycete)	Phytophthora blight	No effect	**(Sharma et al., 2015)**
Chamber – 380 vs 800 ppm $[CO_2]$	*Solanum lycopersicum* – tomato	*Phytophthora parasitica* (oomycete)	Buckeye rot	Decreased severity	**(Zhang et al., 2015)**
Chamber – 380 vs 800 ppm $[CO_2]$	*Solanum lycopersicum* – tomato	*Pseudomonas syringae* (bacterium)	Bacterial speck	Decreased severity	**(Zhang et al., 2015)**
Chamber – 380 vs 800 ppm $[CO_2]$	*Solanum lycopersicum* – tomato	*Botrytis cinerea* (fungus)	Grey mould	Increased severity	**(Zhang et al., 2015)**
Chamber – 380 vs 800 ppm $[CO_2]$	*Solanum lycopersicum* – tomato	*Pseudomonas syringae* (bacterium)	Bacterial speck	Decreased severity	**(Li et al., 2015)**
Chamber – 400 vs 600 vs 800 ppm $[CO_2]$	*Brassica napus* - canola	*Leptosphaeria maculans* (fungus)	Blackleg	Decreased severity	**(Zou et al., 2019)**

[a] Free-air CO_2 enrichment.

1.6 Grey leaf spot disease

Maize is one of the most valuable staple crops worldwide and global demands are predicted to double by 2050 (Leakey et al., 2004, Chaloner et al., 2021). Grey leaf spot is a significant foliar disease of maize that can cause yield losses of up to 70% under favourable environmental conditions. GLS is prevalent in North America, South America and Africa, as it flourishes in humid, warm climates (Latterell and Rossi, 1983, Ward et al., 1999). Both *Cercospora zeae-maydis* and *Cercospora zeina* (Ascomycota) have been identified as the causal agents of GLS, but only the latter has been reported thus far in South Africa (Meisel et al., 2009, Nsibo et al., 2019). *C. zeina* is a foliar fungal pathogen that causes a reduction in the photosynthetic capacity of the maize leaves due to the formation of necrotic lesions that run parallel to leaf venation (Fig. 1). Individual lesions coalesce in severe infections and can result in full foliar blight, which reduces yield by limiting grain filling (Ward et al., 1999, Caldwell and Laing, 2005).

Figure 1. Characteristic mature grey leaf spot lesions on maize at 54 days post inoculation as observed under chamber conditions. Scale bar = 2 cm.

GLS conidia are dispersed via wind or rain droplets, with plant litter and neighbouring infected plants serving as sources of inoculum for secondary infections (Ward et al., 1999, Kim et al., 2011b). Fungal conidia only germinate under highly humid conditions and tend to localise to the abaxial leaf surface. The conidia enter via plant stomata, whereby appressoria form over the leaf openings and hyphae extend intercellularly, resulting in tan, rectangular lesions (Beckman and Payne, 1982, Kim et al., 2011a). The latent period for GLS is approximately 14-28 days from infection to sporulation and the onset of symptoms. The characteristic lesions turn grey upon conidiation, whereby conidiophores arise out of the stomata (Kim et al., 2011b). GLS disease management strategies include tillage practices, crop rotation and the use of fungicides. Developing resistant maize hybrids presents the most effective solution to GLS and quantitative resistance through breeding strategies utilising quantitative trait loci (QTL) have been documented (Zhang et al., 2012, Berger et al., 2014, Zuo et al., 2015, Welgemoed et al., 2020).

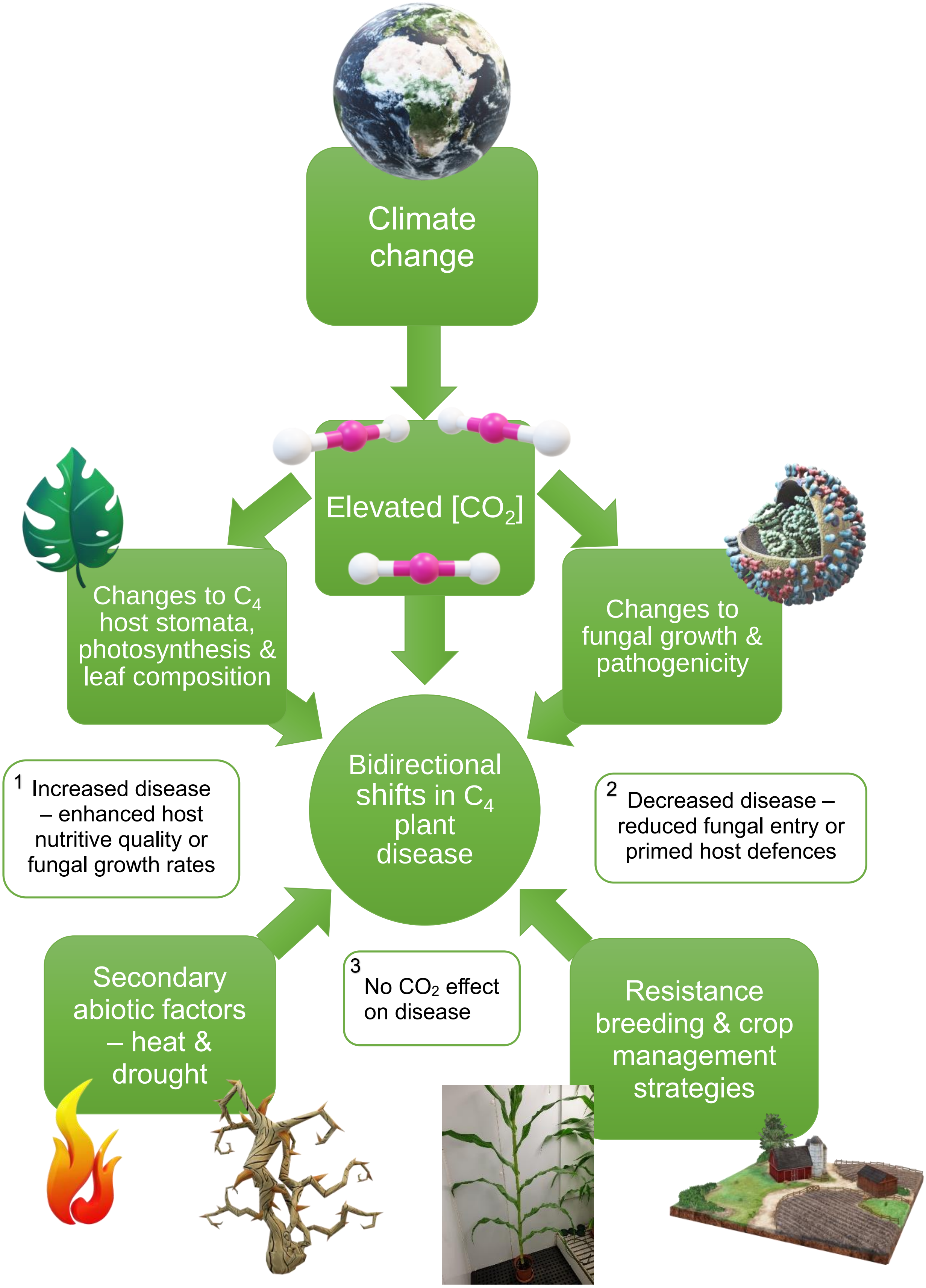
1.7 Conclusions

The findings presented in this literature review indicate that elevated [CO_2] will undoubtedly influence a variety of physiological, molecular and genetic parameters for both C_3 and C_4 plants, as well as fungal pathogens. However, the evidence suggests that the nature of this influence may be specific to the plant and pathogen species being examined. Maize is the most important C_4 crop in the world and has been studied relatively well in CO_2-rich environments. Despite some inconsistency in these trials, certain patterns are discernible, which includes reduced stomatal conductance and growth stimulation under water limiting conditions. Modifications to photosynthetic enzymes, leaf chemistry and gene regulation are also to be expected due to CO_2 enrichment. On the other hand, the fungal response to elevated CO_2 levels remains poorly characterised, but changes in growth, life cycle and the regulation of genes involved in CO_2 perception are common themes. Plant-pathogen interactions are particularly complex to predict at elevated [CO_2] due to the compounding effects on the individual participants. The infection process and dynamic between the host and pathogen is a key factor determining whether disease severity surges or declines. A decrease in disease severity in high CO_2 conditions is often associated with the pathogen's mode of entry, particularly for foliar pathogens that penetrate through host stomata. On the other hand, enhanced nutritional quality of the plant can increase disease development by providing more nutrients for proliferating microorganisms. Elevated [CO_2] are just one factor of climate change and will be accompanied by changing rainfall patterns, rising temperatures and an increase in other greenhouse gases. These potential abiotic stresses will affect plants, pathogens and disease development, as reflected by the ameliorating effect high CO_2 levels have on drought stressed plants and the temperature dependent functionality of enzymes. Adaptation to changing climatic conditions will also influence disease progression, in terms of the evolutionary arms race between host resistance and pathogen fitness.

There is a relatively high degree of variation in some of the findings presented in this literature review, which can be explained by the differences in study design. Depending on the climate projections used, researchers have selected a wide range of 'ambient [CO_2]' (280-450 ppm) and 'elevated [CO_2]' (500-4000 ppm) for their chamber and open field (FACE) experiments. Enclosed chamber conditions can mimic field conditions reasonably well, but there are still limitations, such as less rooting volume and abnormal atmospheric coupling, which can skew results. Most of the FACE trials discussed in this review utilised an elevated [CO_2] of approximately 550 ppm, which corresponds to mid-century prediction models, as opposed to the end of century forecast (800-1000 ppm CO_2). It is possible that a threshold effect could occur, whereby a minimum CO_2 concentration is required to induce a significant CO_2 effect that would not be observed in the aforementioned FACE studies (Leakey et al., 2006). Additionally, differences in cultivar choice, growth conditions (photoperiod and PAR intensity, fertilizer and irrigation regime), start and duration of CO_2 exposure, as well as the timing and nature of measurements, can all contribute to contrasting outcomes. Another shortcoming of research in this field is that many elevated [CO_2] studies on C_4 species were conducted under conditions that are optimal for C_3 plants rather than the high temperature and light conditions that favour C_4 photosynthesis (Maroco et al., 1999). Disease management strategies will need to take the changing environment into account, with regards to plant breeding, crop management and weather monitoring practices, as well as novel technological developments and policy-making. The collective findings in the literature establishes that significantly more research is required on C_4 species and plant-pathogen interactions at elevated CO_2 concentrations, while also accounting for predicted deviations in other biotic and abiotic variables. Furthermore, more consistency in study design is necessary to make meaningful comparisons and conclusions in order to maintain food security, maximise plant products and safeguard natural ecosystems in the projected climate.

Future work to be conducted includes open field disease trials, testing a broad range of [CO_2] to determine any possible threshold effects, as well as the analysis of different C_4 species and maize cultivars. Plant diseases need to be assessed under multiple and interactive abiotic stresses predicted to coincide with elevated CO_2 concentrations, such as drought, heat stress or fluctuations in other greenhouse gases. Disease trials and physiological measurements should be conducted under the elevated radiation and temperature conditions that favour C_4 photosynthesis and are more comparable to natural settings. Chakraborty and Datta (2003) reported a significant increase in *C. gloeosporioides* fecundity and aggressiveness over several infection cycles at elevated [CO_2]. Therefore, long term trials that account for multiple infection cycles should also be performed, as microorganisms are known to evolve faster than plants and could thus elicit worsening effects over time (Coakley et al., 1999). Metagenomics or ancient DNA, such as those in Greenland ice cores, should be studied to ascertain whether fungal populations shifted at varying CO_2 levels throughout history. Lastly, the individual pathogens' responses to CO_2 enrichment should also be investigated via *in vitro* analysis. Chapter 2 below aimed to elucidate the effects of elevated [CO_2] on GLS disease of maize and concluded that no significant difference in disease severity occurred between the 415 and 700 ppm CO_2 regimes.

1.8 Graphical abstract



Climate change

Elevated [CO_2]

Changes to C_4 host stomata, photosynthesis & leaf composition

Changes to fungal growth & pathogenicity

Bidirectional shifts in C_4 plant disease

[1] Increased disease – enhanced host nutritive quality or fungal growth rates

[2] Decreased disease – reduced fungal entry or primed host defences

Secondary abiotic factors – heat & drought

[3] No CO_2 effect on disease

Resistance breeding & crop management strategies

1.9 References

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Chapter 2

Effect of elevated CO_2 on maize susceptibility to grey leaf spot disease

Abstract

Atmospheric CO_2 concentrations have been rising considerably over the last century and are forecast to reach 800-1000 ppm by 2100. Maize is an important C_4 crop that is cultivated around the world; however, its production is subjugated to yield losses due to *Cercospora zeina*-induced grey leaf spot (GLS) disease. The aim of this study was to investigate the effect of elevated [CO_2] on maize susceptibility to GLS across two growing seasons. Maize plants were grown at 415 and 700 ppm CO_2 in enclosed growth chambers and there was no difference in plant height-based growth rates of the B73 maize between CO_2 regimes in either trial. Gas exchange parameters were assessed, and increased leaf photosynthetic rates were observed for maize grown at elevated CO_2 levels in both trials, while greater stomatal conductance was measured on the high CO_2-grown plants in the second trial only. Maize leaf stomatal density and *in vitro C. zeina* growth were shown to be unaffected by elevated [CO_2], along with maize leaf sugars, such as glucose and sucrose. However, maltose concentrations were significantly greater at 415 ppm than 700 ppm CO_2. Maize leaves were inoculated with *C. zeina,* and disease development was recorded and quantified, wherein no CO_2 effect was documented in the first trial. Significantly greater GLS symptoms were observed on plants grown at elevated [CO_2] than ambient in the repeat trial, although this was a consequence of limited GLS development in the ambient CO_2-grown maize. Analysis of the environmental data from each chamber suggests that a chamber effect occurred in the second trial due to relatively lowered humidity in the ambient CO_2 chamber during the critical period for *C. zeina* infection following inoculation. Therefore, the conclusion is that maize susceptibility to GLS will not be significantly affected by elevated [CO_2], which demonstrates a neutral effect of climate change on one major maize disease.

Keywords:
corn, *Cercospora zeina*, gray leaf spot, climate change, gas exchange

2.1 Introduction

Current atmospheric [CO_2] are around 415 ppm and are predicted to at least double by the end of the century (IPCC, 2014). Altered environmental conditions can impact host and pathogen biology and could conceivably change the current landscape of plant disease. Maize (*Zea mays* L.) is an important cereal crop that is grown and consumed worldwide and is particularly valuable in Southern Africa. It is a C_4 species that forms a staple portion of most diets and is commonly utilised for fuel and feed (Young and Long, 2000). However, maize production is subject to yield losses due to pathogen attack, with grey leaf spot (GLS) disease causing a reduction in yield of up to 70% (Ward et al., 1999). GLS thrives in warm, humid climates and has been a cause for concern in North America, South America and Africa (Latterell and Rossi, 1983, Ward et al., 1999). GLS is caused by either fungal pathogens *Cercospora zeina* or *Cercospora zeae-maydis,* with the former being the only causal agent reported to date in South Africa (Meisel et al., 2009, Nsibo et al., 2019). GLS is a foliar disease resulting in tan, rectangular lesions that run parallel to leaf venation and reduce the photosynthetic capacity of the leaves (Ward et al., 1999). *C. zeina* tends to localise to the lower surfaces of maize leaves, with appressoria forming over stomata 4-5 days post inoculation (dpi) and is followed by hyphal penetration 6-7 dpi that is promoted by high levels of relative humidity (Beckman and Payne, 1982). *C. zeina's* response to elevated [CO_2] is currently unknown and potential changes in the specific dynamic between the fungus and maize could positively, negatively or neutrally affect GLS disease development and severity.

Leaves undergo many physiological and morphological changes in response to elevated CO_2 levels, which may be reflected by fluctuating gas exchange and altered stomatal development. Gas exchange parameters, such as the photosynthetic rate (*A*) and stomatal conductance (g_{sw}), provide insight into the inner functioning of plant systems and their response to different environments. Photosynthesis is a measure of maize leaf productivity and relates to the amount of plant carbohydrates produced, while correspondingly being directly reduced by GLS infection. On the other hand, stomatal conductance is positively correlated with stomatal opening, which in addition to stomatal density (SD), could influence *C. zeina's* penetration or infection rate due to the pathogen's mode of entry. Elevated [CO_2] can alter *in vitro* fungal growth, as McElrone et al. (2005) showed that the foliar pathogen *Phyllosticta minima* grew faster in high CO_2 conditions than ambient on PDA. Increased pathogenicity has been observed in response to elevated CO_2 levels in several ascomycetes, including *Magnaporthe oryzae, Erysiphe cichoracearum*, *Fusarium verticillioides, Fusarium oxysporum* and *Botrytis cinerea* (Kobayashi et al., 2006, Lake and Wade, 2009, Vaughan et al., 2014, Chitarra et al., 2015, Zhang et al., 2015). On the other hand, a decrease in pathogenicity has been reported for *Erysiphe graminis*, *Colletotrichum gloeosporioides* and *P. minima* due to enriched [CO_2] (Hibberd et al., 1996b, Chakraborty et al., 2000a, McElrone et al., 2005). Very few studies have been conducted on maize diseases at elevated CO_2 concentrations and none on GLS. It is vital that farmers can sustainably maintain maize production in the predicted future climate and understanding the response to a destructive disease is a necessary step.

The aim of this study was to evaluate the interaction between *C. zeina* and host maize plants at elevated CO_2 concentrations across two independent trials. Susceptible B73 maize line plants were grown at ambient (415 ppm CO_2) and elevated [CO_2] (700 ppm) in separate Conviron growth chambers. *C. zeina* inoculum was prepared and half of the plants in each chamber were inoculated, while the other half of control plants were mock-inoculated. The effects of CO_2 enrichment on maize and *in vitro* fungal growth were also assessed individually. Maize leaf photosynthetic rates, stomatal conductance, stomatal density and metabolite concentrations were appraised between the different treatments. GLS symptom development and pathogenicity was scored visually and *in planta* fungal content quantified using a qPCR assay. The null hypothesis (H_0) was that there would be no significant difference in GLS disease development between ambient and elevated CO_2 treatments. The alternate hypothesis (H_1) predicted that lower disease development would occur in maize plants cultivated at elevated CO_2 levels versus ambient due to enhanced stomatal closure and reduced fungal load.

2.2 Materials and methods

2.2.1 Pilot GLS disease trial

A pilot study was first performed in one PGCFlex Conviron growth chamber (ID 005) (Conviron, Winnipeg, Canada) to select a suitable maize line, as well as establish an optimal maize growth and *Cercospora zeina* inoculation protocol. B73 inbred line and PAN 5A-154 hybrid maize plants were grown under ambient CO_2 levels (415 ppm) and inoculated with a *C. zeina* (CMW 25467, Mkushi) conidial suspension at the V5, V6 and V7 leaf stages using the paintbrush method. The respective maize lines were selected due to their susceptibility to GLS disease (Christie et al., 2017, PANNAR, 2019). The growth chamber was controlled at 28°C day/ 16°C night, 1125 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), 12-hour photoperiod, 40% relative humidity raised to 100% (RHmax) post inoculation (for one week) and 415 ppm [CO_2]. Single maize plants were sown in 24 (12 per maize line) circular, plastic pots (22 cm diameter x 24 cm height) containing a silica sand and Culterra® seedling mix (Culterra, Nietgedacht, South Africa). Planting occurred over the course of three weeks, with planting dates on the first day of each week (8 per week). All the plants were inoculated and mock-inoculated on the same day at the respective leaf stages. Three maize leaves per plant were inoculated with a consistent inoculum concentration of 9.2×10^5 conidia/ml on both the adaxial and abaxial surfaces. Control plants were mock-inoculated with 0.02% Tween 20 and a vertically hanging transparent plastic sheet was inserted in the Conviron to separate the respective treatments. Disease symptom development was photographed and visually scored every four days on a S1-S6 scale until 42 dpi. The results from the pilot study were used to design the main experimental trials.

2.2.2 Plant material and growth conditions

B73 maize plants were grown under ambient CO_2 levels (415 ppm) in one PGW40 Conviron growth chamber (ID 001) (Conviron, Winnipeg, Canada) and under elevated [CO_2] (700 ppm) in a separate PGW40 chamber (ID 002). The experiment was performed twice, and the same chambers were used for the respective treatments in each trial. A transparent plastic barrier was installed in each Conviron to separate the inoculated and mock-inoculated plants. Each chamber housed 40 single maize plants in plastic pots (20 cm diameter x 24 cm height). The growth chambers were controlled at the following conditions, which simulate a characteristic maize growing season and region (summer in Cedara, Pietermaritzburg): 28°C day/16°C night, 1400 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD), 13-hour photoperiod, 60% relative humidity increased to 100% (RHmax) for 2 weeks post inoculation, and the respective [CO_2] (415 or 700 ppm) (AccuWeather, 2020).

A silica sand: Culterra® seedling mix (Culterra, Nietgedacht, South Africa) (1:1 ratio (m/m)) was used as plant growth media. The plants were watered three times per day (7 am, 11 am and 4 pm) with 200-300 ml drip irrigated into the pots at each instance. Hygroponic® fertiliser was used to supplement the maize by gradually adding increasing concentrations of the water-soluble mixture every second day, starting with 1 g/L four days after emergence (dae) and incrementally moving up to 4 g/L by the silking stage. Maize growth and development were recorded once a week, whereby the leaf stage (V-stage) was counted using the leaf collar method (Purdue University, USA, https://extension.entm.purdue.edu/fieldcropsipm/corn-stages.php) and plant height (cm) was measured from soil base to leaf tip.

2.2.3 *In vitro C. zeina* growth

C. zeina was grown *in vitro* at both ambient and elevated CO_2 levels in constant darkness in Tupperware® containers placed in the corresponding growth chambers. Single colonies were prepared by diluting a fungal suspension prepared from 7-day old Mkushi (CMW 25467) cultures grown on V8 media in constant darkness to promote sporulation. The experiment was repeated, with 750 colonies pipetted onto each V8 plate for the first experiment and 250 colonies for the second. The plates were sealed with gas permeable micropore tape (3M, Minnesota, USA) to prevent contamination, but allow CO_2 inflow. The plates were photographed every 3-5 days using a digital camera (Samsung Galaxy Note 9, Seoul, South Korea). Colony size (mm^2) and number were determined using ImageJ (Fiji) software (US National Institutes of Health, Maryland, USA).

2.2.4 Maize inoculation with *C. zeina*

The paintbrush method was used to inoculate maize leaves with *C. zeina* at the V6 leaf stage (Meisel et al., 2009). The plants were inoculated at 43 days after planting (dap) for trial 1 and 48 dap for trial 2. *C. zeina* isolate CMW 25467 (Mkushi) inoculum was prepared by first growing the fungus on V8 agar medium (800 ml of distilled water, 200 ml of V8 juice, 15 g of agar, 2 g of $CaCO_3$) supplemented with 50 µg/ml of cefotaxime at 25°C in constant darkness. Inoculum was prepared from 7-day old plates, whereby conidia were rinsed with 0.02% Tween 20 and dislodged with a steel spatula to attain an inoculum concentration of 7.8×10^5 conidia ml^{-1} for trial 1 and 8.8×10^5 conidia ml^{-1} for trial 2 (Table S1). A 15 cm long section in the middle region of each leaf was demarcated with black marker and the inoculum was generously applied onto both the adaxial and abaxial sides of the three most recent fully developed leaves (V4-V6 stages) per maize plant with a small sterile paintbrush. Control plants were treated with 0.02% Tween 20 in an identical manner. The relative humidity (RHmax) was set to 100% in both chambers for 14 dpi to stimulate spore germination.

Photographs of the 15 cm long demarcated area of the inoculated and mock-inoculated leaves were taken approximately every four days until 46 dpi (reproductive stage) with a digital camera (Samsung Galaxy Note 9, Seoul, South Korea). GLS disease symptoms were scored visually on a S1-S6 scale on 10 representative biological replicates per treatment (Table 4).

Table 4. Disease scale for visually recording GLS disease development.

Disease Score	Description	Symptom Representation
S1	no symptoms	
S2	chlorotic spots	
S3	chlorotic lesions	
S4	few single lesions (2-8)	
S5	several single lesions (9-15)	
S6	coalesced lesions	

2.2.5 Gas exchange measurements

Net CO_2 assimilation (*A*) (photosynthetic rate) and stomatal conductance (g_{sw}) were measured on the inoculated and mock-inoculated leaves, as well as the most recent fully expanded leaf, using a LI-6800 portable photosynthesis system (Li-COR, Nebraska, USA) equipped with the 6800-01 fluorometer. The measurements were taken between 10:00 am and 16:00 pm and the growth chamber conditions were kept consistent. The gas exchange parameters were measured at four different stages of disease development for both trials: yellow chlorotic spot formation (21 dpi), yellow chlorotic lesion formation (28 dpi), elongated tan single lesions (33-

34 dpi) and mature coalesced lesions progressing to severe blight (41 dpi). The control plants served as a source of symptomless maize leaf material.

The LI-6800 system was warmed up, calibrated, and set to the appropriate configuration settings corresponding to the chamber conditions. The saturating irradiance and flow rate were set to 1000 µmol $m^{-2}s^{-1}$ and 500 µmol mol^{-1}, respectively. The [CO_2] in the leaf chamber was equilibrated to the Conviron concentration (415 or 700 ppm) by the LI-6800 CO_2 mixer and the temperature adjusted to that of the growth chambers at the time of taking the measurements. Representative maize leaves (middle region of one side of the midrib of the demarcated 15 cm long area) from each CO_2 and inoculation treatment were non-destructively placed in the centre of the gas analyser's leaf chamber. The gas exchange measurements were then logged manually using stability/matching criteria for net CO_2 assimilation, stomatal conductance, and transpiration to one standard deviation and slope limit over a period of 15 seconds. All data were collected and analysed using the associated software for the Li-COR system.

2.2.6 Stomatal density counts

Stomatal density (SD) was assessed in a separate, but identical, GLS disease chamber trial at four days prior to inoculation (65 dap). Stomatal density was measured on the three ranks of inoculated/mock-inoculated leaves (V4-, V5- and V6-stage), as well as the most recent fully expanded leaf (V9-stage), per plant. Stomatal impressions were prepared by applying clear nail polish to a central section parallel to the midrib of both the adaxial and abaxial leaf surfaces. Transparent tape was used to transfer the 3x2 cm imprints onto glass microscope slides. The epidermal peels were examined using a Nikon Eclipse E200 light microscope (Nikon Corporation, Tokyo, Japan) at 100X magnification with a field of view (FOV) of 2 mm (1.78). The micrographs were imaged with an equipped Olympus EP50 camera (Olympus Corporation, Tokyo, Japan). Stomatal numbers were manually counted on randomly selected digitized micrographs from six sections of the upper and lower surfaces. The area of FOV and stomatal density were calculated as follows: area of FOV = π x radius squared; SD = number of stomata in FOV/area of FOV.

2.2.7 Metabolite analysis

Selected primary metabolites, namely glucose, fructose, sucrose, maltose and trehalose, were analysed using various corresponding sugar assays, according to Griffiths et al. (2020). Sample material was collected at three different time points (34, 42 and 55 dpi) during trial 1 only due to time constraints. All leaf material harvested was placed in foil packets, flash frozen in liquid nitrogen and stored at -80°C before being freeze dried and sent for metabolite analysis at Rothamsted Research.

To evaluate the concentration of hexose sugars (glucose, fructose and sucrose) in maize leaves, samples were ground to a fine powder and 100-150 mg of tissue was extracted in 80% ethanol for 2 hours while shaking at 80°C. Cooled samples were then centrifuged at 12000 *g* for 5 min to remove debris. A 5 µl aliquot of sugar extract was subjected to coupled enzyme assay to determine glucose, fructose and sucrose concentrations. The enzyme assay was conducted at room temperature in a HEPES-based buffer (100 mM HEPES, 4 mM $MgCl_2$, 1 mM NAD+, 0.5 mM ATP) at pH 7.4 using 10 µl of ethanolic sugar extract. Enzymes were sequentially added to determine the concentration of each hexose sugar. Glucose and fructose were measured 30 min following sequential enzyme addition, while sucrose was measured 1.5 hours after enzyme addition. Firstly, glucose was determined by the addition of 1.5U hexokinase and 1.2U NAD-dependent glucose-6-phosphate dehydrogenase. Secondly, fructose was measured after the addition of 0.2U of phosphoglucoisomerase, and lastly, sucrose was measured following the addition of 10U invertase. Measurements of each sugar were completed on a microplate reader (Spectramax, Molecular Devices, California, USA) by measuring the reduction of NAD+ to NADH at 340 nm. The concentration of each sugar per sample was determined using a standard curve and standardised to the weight of tissue extracted in mg (dry weight).

Maize leaf tissue was extracted as per the hexose sugars to quantify maltose and trehalose levels. A 20 µl aliquot of sugar extract was subjected to coupled enzyme assay to determine maltose and trehalose contents. The enzyme assay was conducted at room temperature in a HEPES-based buffer at pH 7.4 using 10 µl of ethanolic sugar extract. For the trehalose assay, 2 µl of purified *Escherichia coli* Trehalase F, 1.5U hexokinase and 1.2U NAD-dependent glucose-6-phosphate dehydrogenase were added. Measurements were completed every hour on a microplate reader by assessing the reduction of NAD+ to NADH at 340 nm. Similarly for maltose determination, 2U of α-D-glucosidase was used in place of Trehalase F, while the rest of the assay was conducted as described above. Concentrations of each sugar per sample were determined using a standard curve and standardised to the dry weight of tissue extracted in mg.

2.2.8 *In planta C. zeina* quantification

The cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA (gDNA) from both plant and fungal tissue (Möller et al., 1992). gDNA was extracted from the inoculated and mock-inoculated maize leaf samples harvested at 29-30 dpi (chlorotic single lesions) and 42-44 dpi (mature coalesced lesions) for both trials, and additionally at 23 dpi (chlorotic spots) for trial 2. *C. zeina* gDNA was extracted from cultures grown on cellophane for 10 days. The integrity and quality of the DNA was analysed using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Massachusetts, USA) at 260 nm, as well as via gel electrophoresis. The DNA samples were diluted to 10 ng/µl with sterile distilled water prior to use in PCR.

The amount of fungal DNA was quantified *in planta* using a quantitative PCR (qPCR) assay following methods described in Korsman et al. (2012). Two sets of primer pairs, which were obtained from laboratory stock solutions, were used for quantification with one pair specific to *C. zeina* and the second pair specific to maize for normalisation purposes. The first set amplifies a putative *C. zeina* cytochrome P450 reductase gene [CPR1_2F (5′-TGAACTACGCGCTCAATG-3′) and CPR1_2R (5′-TCTCTCTTGGACGAAACC-3′)], while the second pair amplifies a maize glutathione *S*-transferase III gene [GST3F (5′-GGAGCCCTGAGTCGAATAAAAG-3′) and GST3R (5′-AACACACACGAAAGGCAACAGT-3′)].

Conventional PCR was performed to ensure that a single product was amplified for each primer pair; a 164 bp *cpr1* fragment from *C. zeina* gDNA and a 106 bp *gst3* amplicon from maize. The PCR was set up in a total volume of 12 µl and consisted of 1X Ampliqon Taq, 1 µl of each primer (10 µM), 5 µl of sterile distilled water and 1 µl of template DNA (10 ng/µl). The cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 60°C for 15 sec and 72°C for 20 sec, and a final extension step of 72°C for 5 min. The reaction was performed on an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, California, USA) and the PCR products analysed using agarose gel electrophoresis.

The qPCR was performed in triplicate using a Bio-Rad CFX96TM Real-Time PCR detection system (Bio-Rad, California, USA). Each amplification reaction was set up in a total volume of 8 µl that consisted of the following: 1x SsoAdvanced™ Universal SYBR® Green Supermix, 10 µM of each primer, template DNA (10 ng/µl) and sterile distilled water. The cycling conditions were as follows: 95°C for 30 sec, followed by 35 cycles of 95°C for 15 sec and 60°C for 30 sec. In order to determine the qPCR efficiency, a standard curve was generated by plotting $\log_{10}$ of the [DNA] of known standards against the number of cycles at which the fluorescent signal from the amplified PCR products surpassed the detection threshold. The standard curve was prepared across 6-dilution points (50, 25, 12.5, 6.25, 3.125 and 1.563 ng) for the *gst3* primers and 8-dilution points (5, 2.5, 1, 0.75, 0.5, 0.25, 0.1 and 0.075 ng) for the *cpr1_2* primers.

The equation of the linear regression line, along with the correlation coefficient of determination (R^2), were used to evaluate the optimization of the qPCR assay. Melt curve analysis (60-95°C) was performed at the end of the thermal cycle to ensure that a single product was amplified. The qPCR efficiency (%) was calculated as equal to $10^{(-1/\text{slope})}$. Gene quantification between treatments was normalised according to the delta-delta Ct ($2^{-(\Delta\Delta Ct)}$) method by dividing the amount of *C. zeina cpr1_2* by maize *gst3* in the inoculated leaf samples to yield ng *C. zeina* DNA per mg maize DNA. The qPCR data were analysed using qbase$^+$ version 3.0 (Biogazelle, Zwijnaarde, Belgium) and Bio-Rad CFX Maestro version 2.2 (Bio-Rad, California, USA) software. The size and quality of the qPCR products were confirmed via agarose gel electrophoresis.

2.2.9 Statistical analysis

The statistical significance of plant height, gas exchange parameters, stomatal density and *in planta* fungal content between the CO_2 treatments was assessed using a Mann-Whitney *U* test ($P<0.05$). Two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni multiple comparison test was used to evaluate statistically significant effects ($P<0.05$) of the independent factors 'CO_2 concentration' and 'days post inoculation', as well as their interaction on the divergence between sugar levels, disease scores, relative fungal load and *in vitro* fungal growth. GraphPad Prism version 5 (GraphPad Software Inc., California, USA) and qbase$^+$ version 3.0 (Biogazelle, Zwijnaarde, Belgium) software were used to conduct the statistical analyses.

2.3 Results

2.3.1 Pilot GLS disease trial

Maize growth and GLS disease progression were examined in one enclosed PGCFlex Conviron growth chamber at ambient CO_2 (415 ppm). The PAN 5A-154 hybrid maize and inbred B73 plants reached the vertical growth limit (132 cm) of the chamber at 61 and 66 dap, respectively (data not shown). The newly formed leaves and stems were continuously pruned to prevent excessive shading and light damage. The B73 maize line has a relatively slow growth rate that made it suitable for testing in the enclosed chambers, which have limited vertical growth space. The hybrid PAN maize grew more robustly than the B73 plants in the simulated conditions, but its enhanced growth rate and size was not ideal for a chamber trial.

Initial GLS symptoms in the form of yellow chlorotic spots developed at 21 dpi on the inoculated leaves of both maize varieties. However, a greater number of chlorotic and necrotic lesions formed subsequently on the B73 maize leaves than on the PAN hybrids until 42 dpi. No significant difference in disease development was observed between the three ranks (V5, V6 and V7) of inoculated leaves, while the control plants remained symptomless throughout the trial (Fig. S1). B73 is a susceptible maize line and GLS disease development was therefore more readily discernible in comparison to the more resistant PAN 5A-154 maize. The V6 leaf stage was determined to be the optimal growth stage for inoculation, as it allowed for sufficient time for symptoms to fully develop within the spatial constraints of the Convirons. In addition, PAN 5A-154 is a hybrid maize line with a heterozygous genotype, which made the prospect of downstream genetic testing more difficult in comparison to homozygous B73 maize. Therefore, the B73 maize line was the ideal candidate to be used in the main trials.

2.3.2 Main experimental trials

2.3.2.1 Maize growth and inoculation

B73 maize plants were grown at 415 and 700 ppm [CO_2] in two different PGW40 Conviron growth chambers (Fig. 2B). Plant height was measured weekly, and no significant difference (Mann-Whitney *U* test, $P>0.05$) in growth was observed between the CO_2 treatments for both trials (Fig. 3). Additionally, the plants in each chamber reached the same V-stage at analogous times. Yellow streaking characteristic of B73 plants grown in enclosed chambers (Swart et al., 2017) was present on some of the maize leaves in both trials. A few plants in both chambers displayed an anthocyanin stress response, as evidenced by purpling stems, and this was overcome by increasing the concentration of fertilizer applied. The B73 plants reached the vertical growth limit of 173 cm in both chambers at 59 dap for trial 1 and 62 dap for the second trial. The newly formed leaves and stems were then trimmed regularly to limit excessive shading. The three most recent collared leaves (V4, V5, V6) (Fig. 2A) were inoculated at 43 (trial 1) and 48 dap (trial 2) with a high concentration ($>7\times10^5$ conidia ml^{-1}) of *C. zeina* conidial spores prepared from bulked up cultures (Fig. 2C).

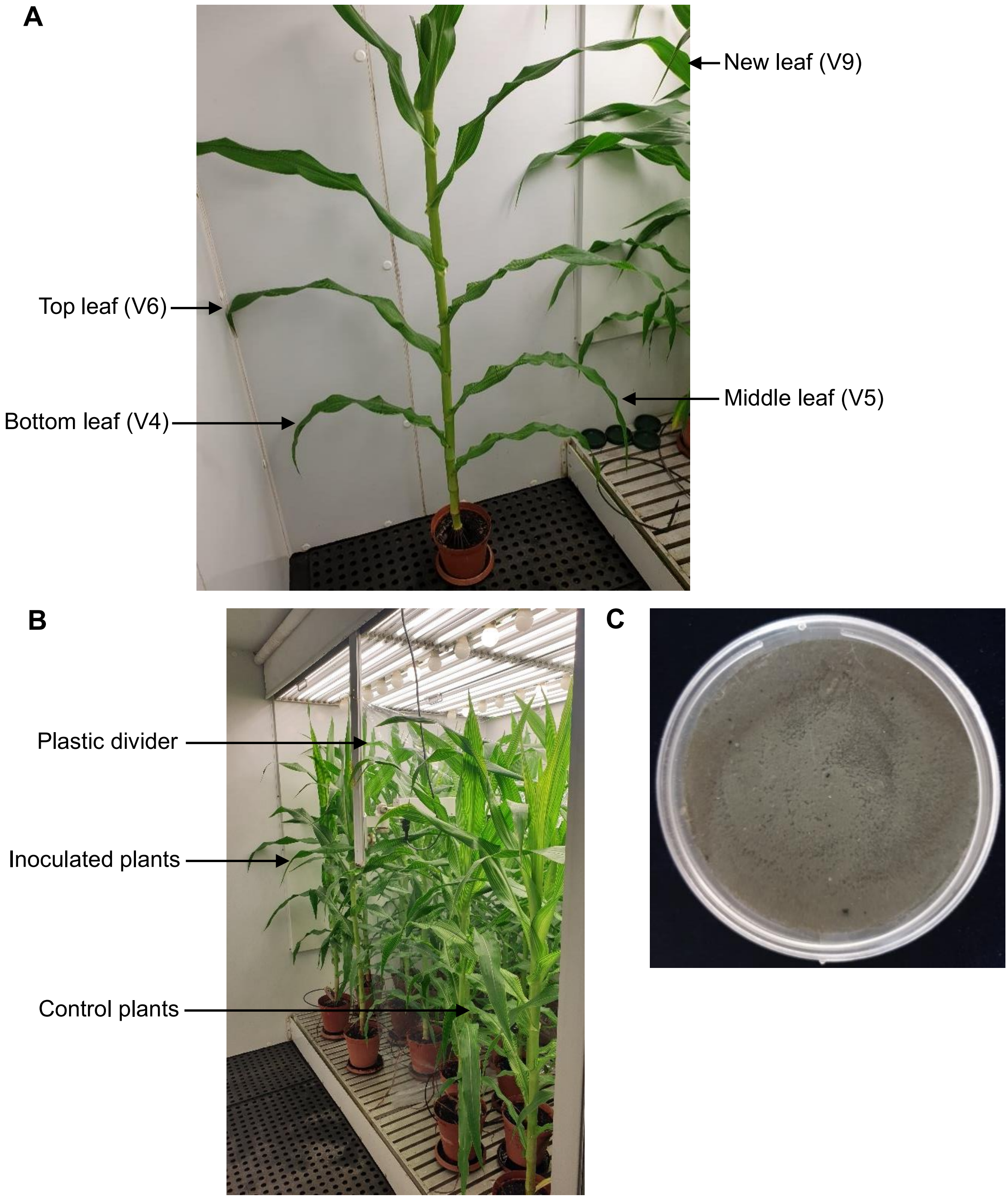

Figure 2. (A) Representative photo of B73 maize growing in a PGW40 Conviron at 29 dpi. The three inoculated/mock-inoculated leaves (V4-V6), as well as a new fully developed leaf (V9-stage), are highlighted. (B) Conviron chamber layout showing inoculated and mock-inoculated maize plants at 13 dpi. (C) Representative photo of *C. zeina* on V8 agar grown in constant darkness showing the characteristic grey colour of sporulating conidia at 8 dpi.

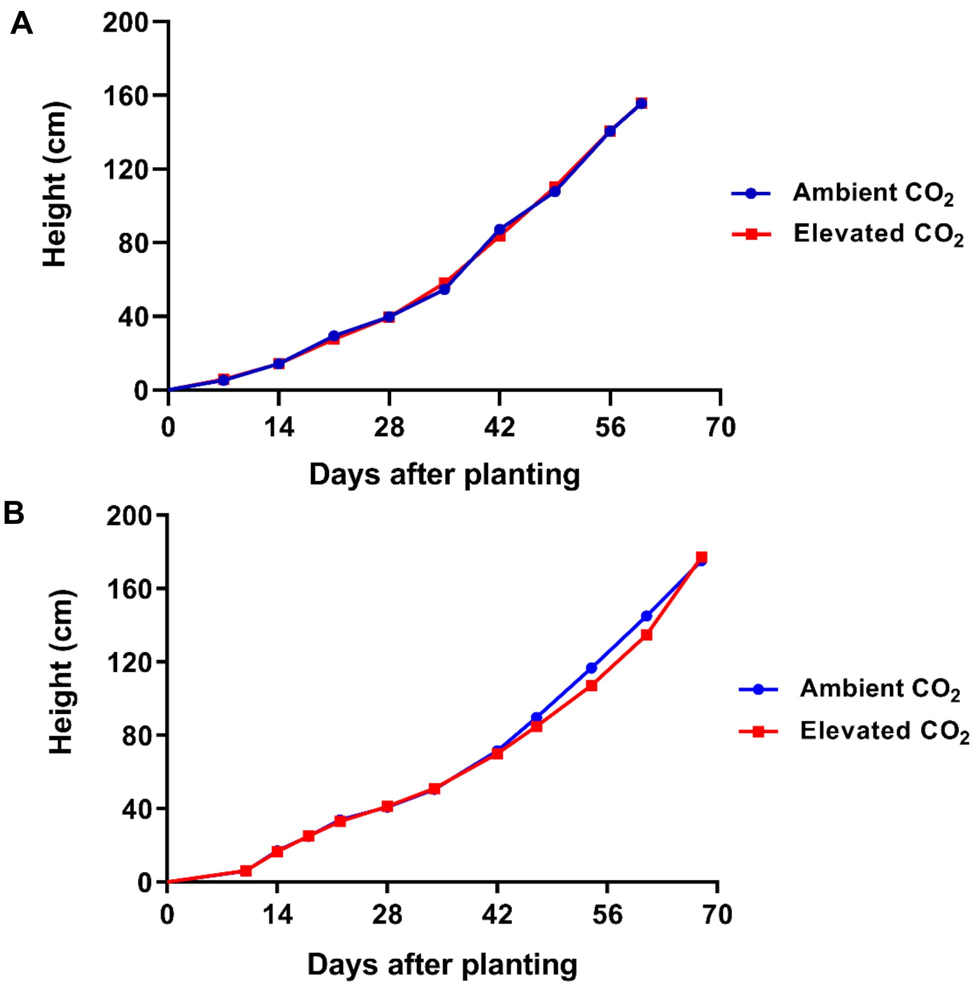

Figure 3. Average growth rate (cm) of B73 maize plants at ambient vs elevated [CO_2] for trial 1 (A) and trial 2 (B). No statistical difference in maize growth (n = 10) was observed between treatments for both trials, based on a paired t test (alpha = 0.05).

2.3.2.2 Growth chamber conditions

The enclosed PGW40 Convirons were programmed to simulate natural maize growing conditions, which were maintained for the duration of both disease trials. Environmental trend data of the growth conditions within each chamber, namely the CO_2 concentration (Fig. 4), relative humidity (Fig. 5), photosynthetically active radiation (PAR) intensity (Fig. 6) and temperature (Fig. 7), were recorded for the entirety of the trials (81-90 days). However, some data points were missing or erroneous due to technical issues with the Convirons, namely faulty gas control valves and defective dehumidifiers. Nonetheless, plant growth conditions were relatively stable for the majority of the trials, with minor deviations between chambers. The period from inoculation to initial symptom formation (0-21 dpi) is the decisive phase that determines GLS disease development due to the direct interaction of the enclosed environment with the susceptible host and pathogen (Fig. 8-11).

In terms of the $[CO_2]$, occasional spikes (3, 7, 12, 13, 14, 17 and 21 dpi) and dips (5, 8, 9 and 10 dpi) in the CO_2 level (ppm) occurred in the elevated CO_2 chamber, while less frequent spikes (8, 9, 16 and 21 dpi) and no dips were recorded for the ambient CO_2 chamber for trial 1 (Fig. 8A). On the other hand, for trial 2, the CO_2 levels were relatively stable and within the range of the setpoint values around the 8-21 dpi period (Fig. 8B). The relative humidity (%) was very similar between the ambient and elevated CO_2 chambers from 0-21 dpi for trial 1 (Fig. 9A). In contrast, the RH during trial 2 was marginally lower in the ambient CO_2 chamber (Fig. 9B), which maintained the lowest relative humidity across Convirons and trials following inoculation (Fig. 5). The PAR intensity readings ($\mu mol\ m^{-2}s^{-1}$) were slightly greater in the 415 ppm CO_2 chamber than the elevated CO_2 Conviron for trial 1 around inoculation (Fig. 10A), while the reverse PAR levels were recorded for trial 2 (Fig. 10B). The Conviron light sensors were affixed at set positions within the chambers (canopy-level) and increased shading occurred due to plant growth, which resulted in declining PAR readings as the trials progressed (Fig. 10). No deviation in the temperature (°C) of the respective chambers was noted for either trial (Fig. 11).

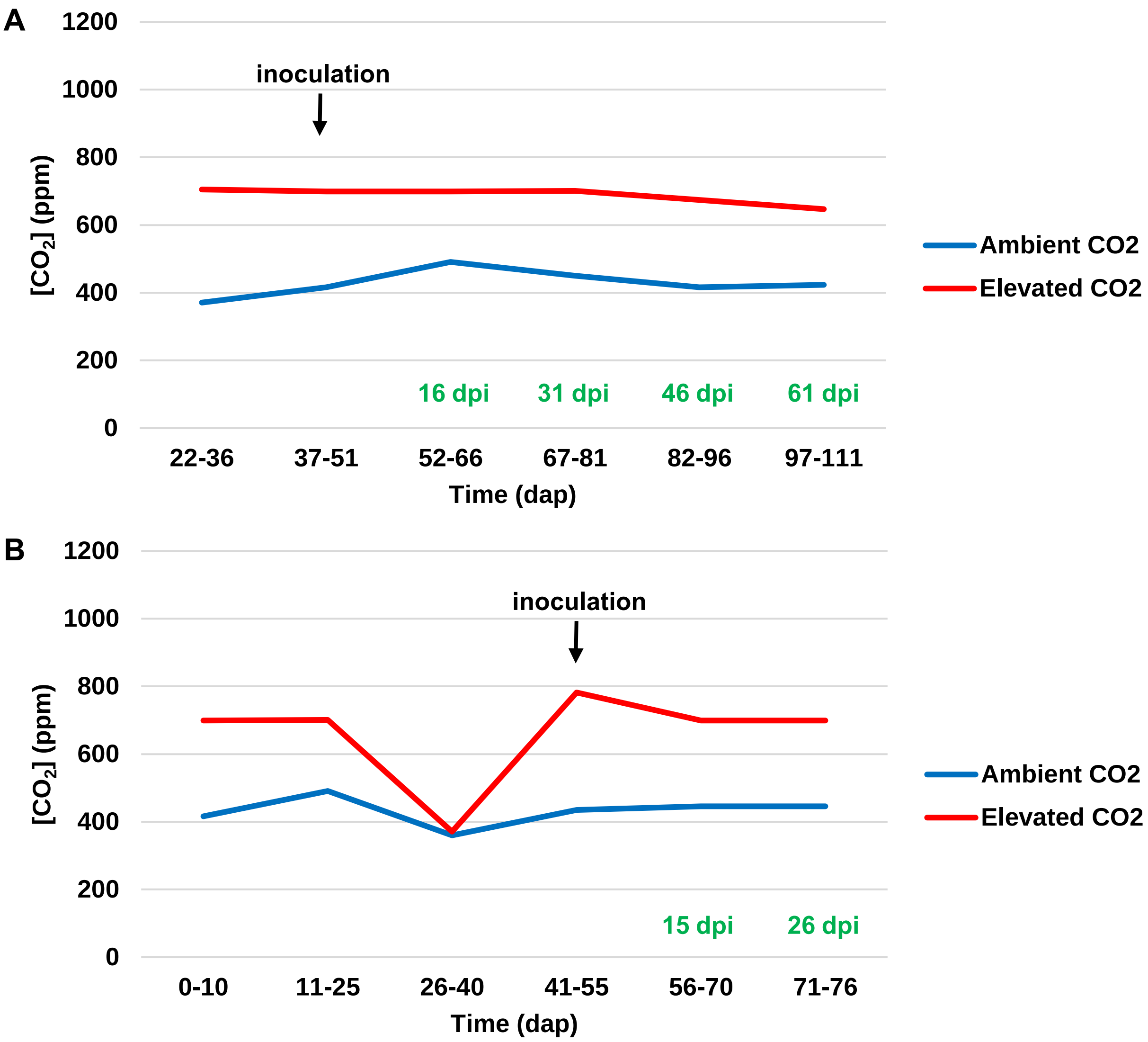

Figure 4. [CO_2] (ppm) in the ambient and elevated CO_2 growth chambers for trial 1 (A) and 2 (B). Median values were plotted per 2-week period. Midpoint days post inoculation (dpi) are shown in green.

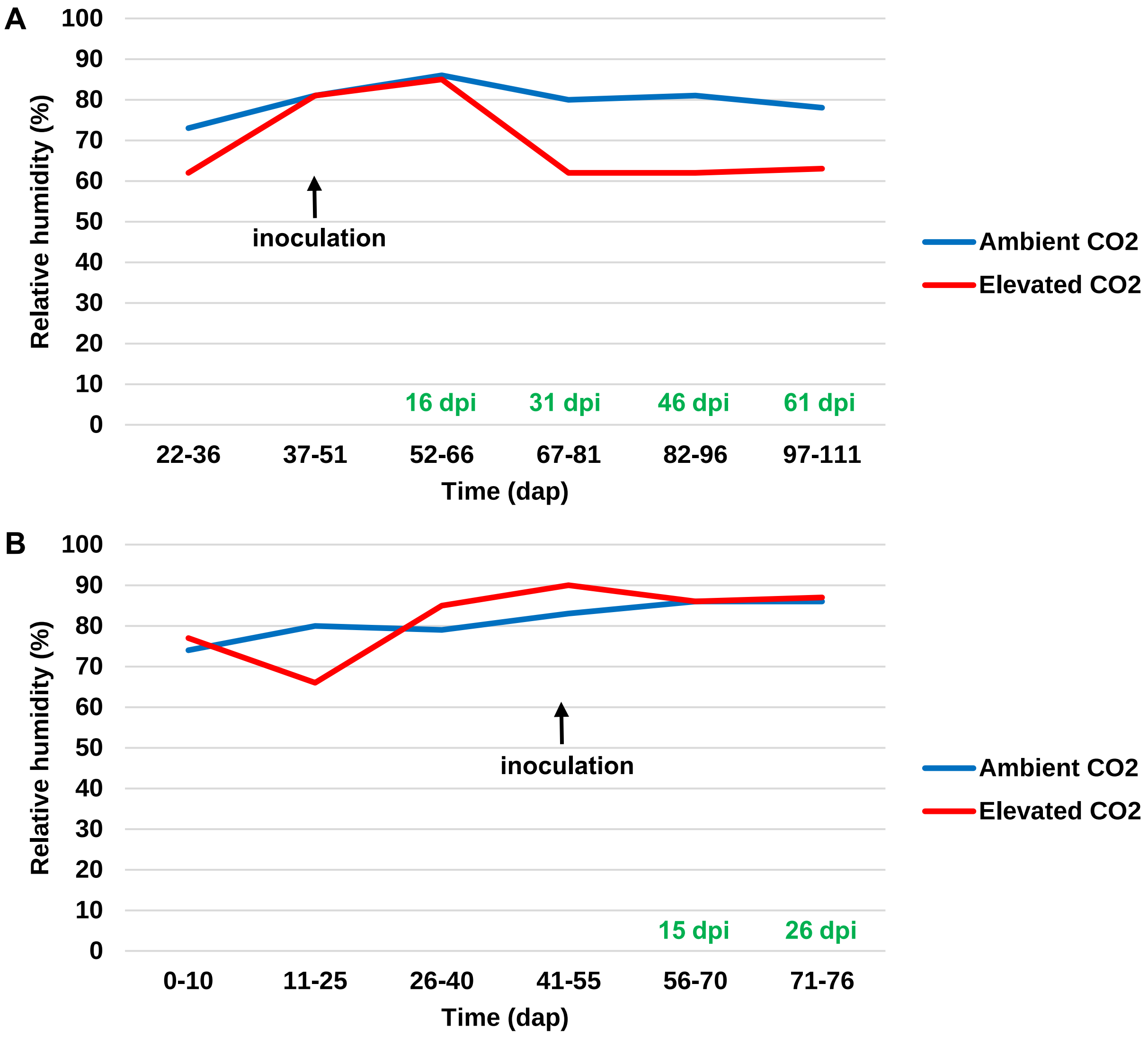

Figure 5. Relative humidity (%) in the ambient and elevated CO_2 growth chambers for trial 1 (A) and 2 (B). Median values were plotted per 2-week period. Midpoint days post inoculation (dpi) are shown in green.

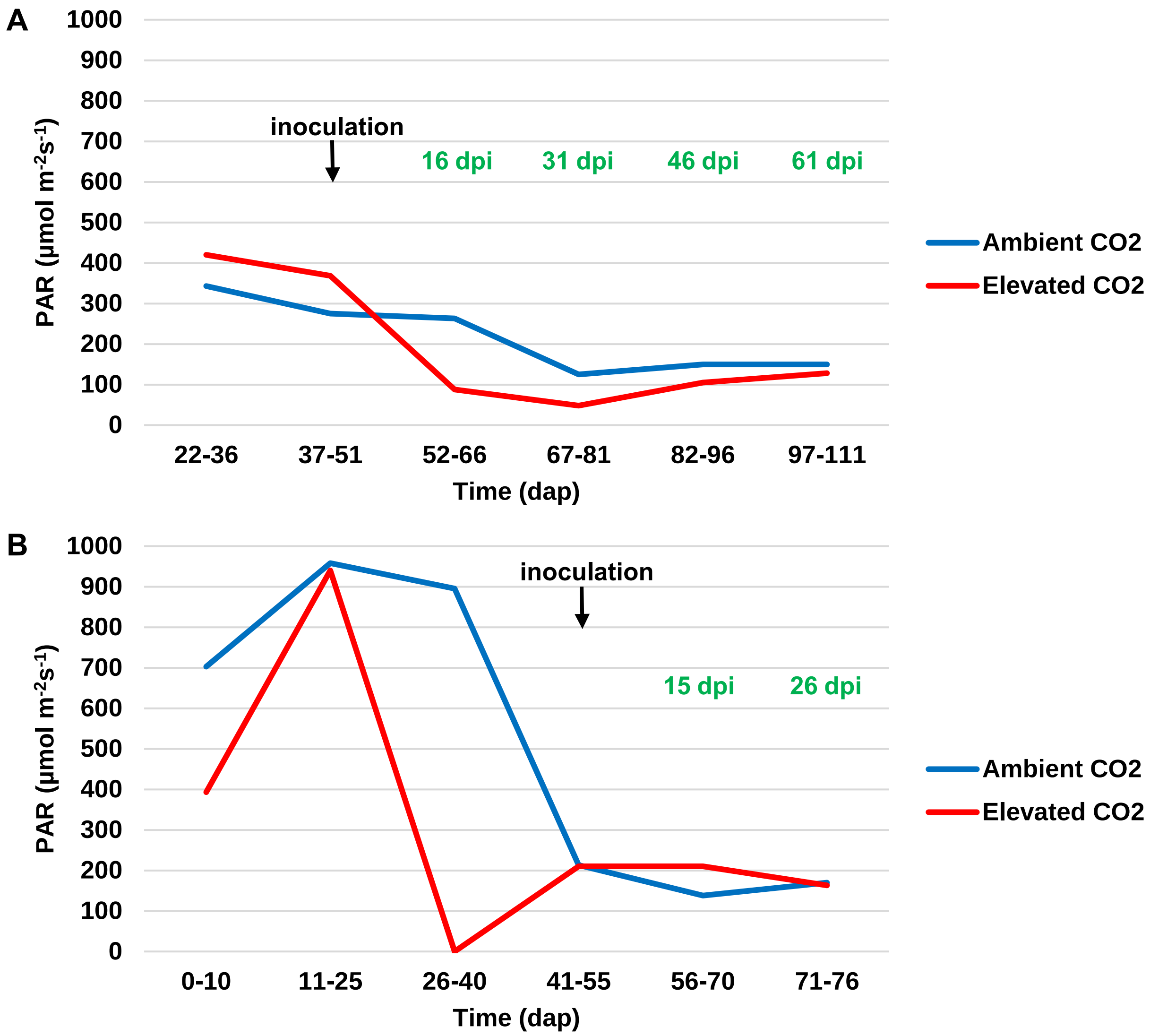

Figure 6. PAR intensity (μmol m^{-2}s^{-1}) in the ambient and elevated CO$_2$ growth chambers for trial 1 (A) and 2 (B). Median values were plotted per 2-week period. Midpoint days post inoculation (dpi) are shown in green.

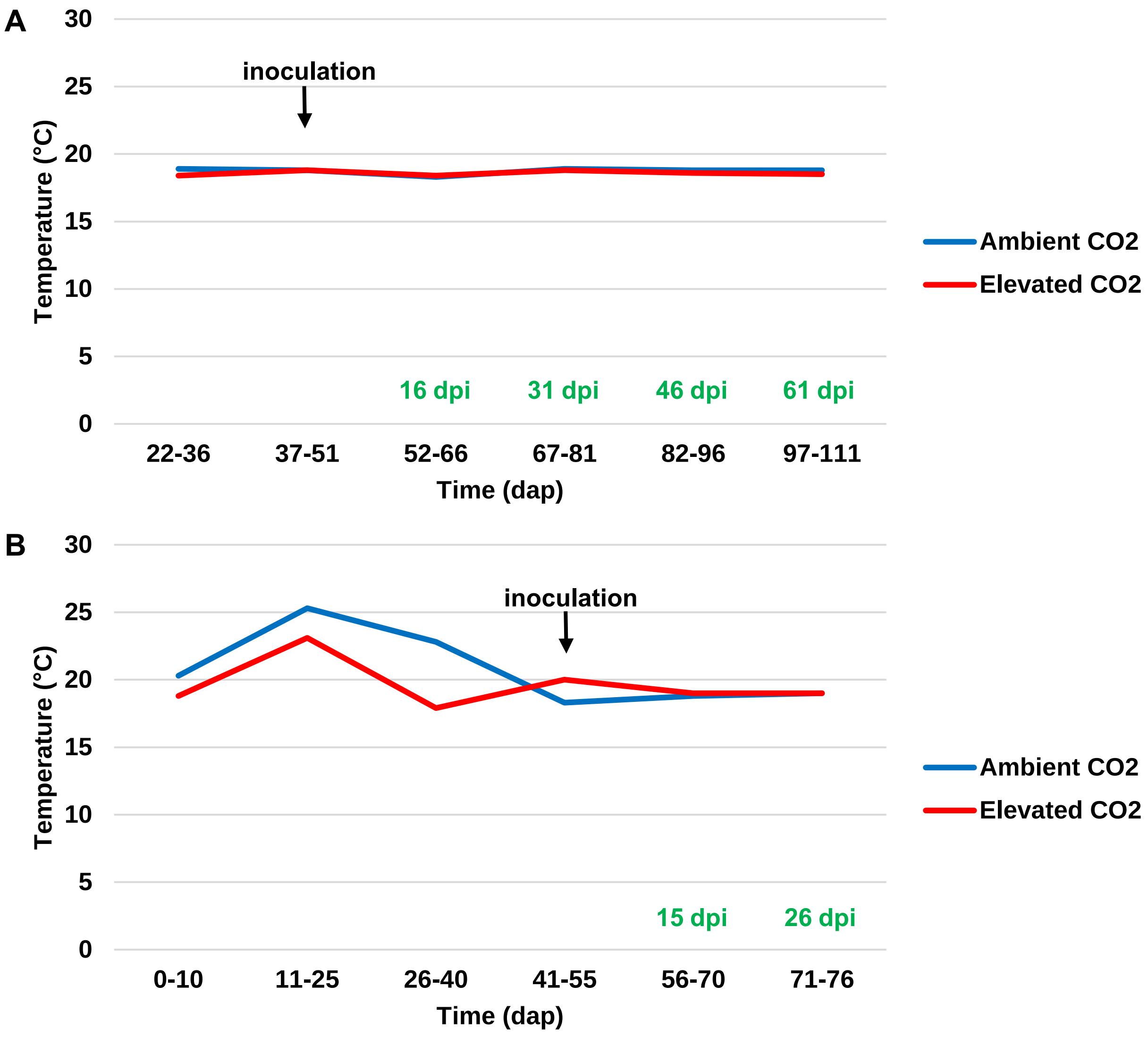

Figure 7. Temperature (°C) in the ambient and elevated CO_2 growth chambers for trial 1 (A) and 2 (B). Median values were plotted per 2-week period. Midpoint days post inoculation (dpi) are shown in green.

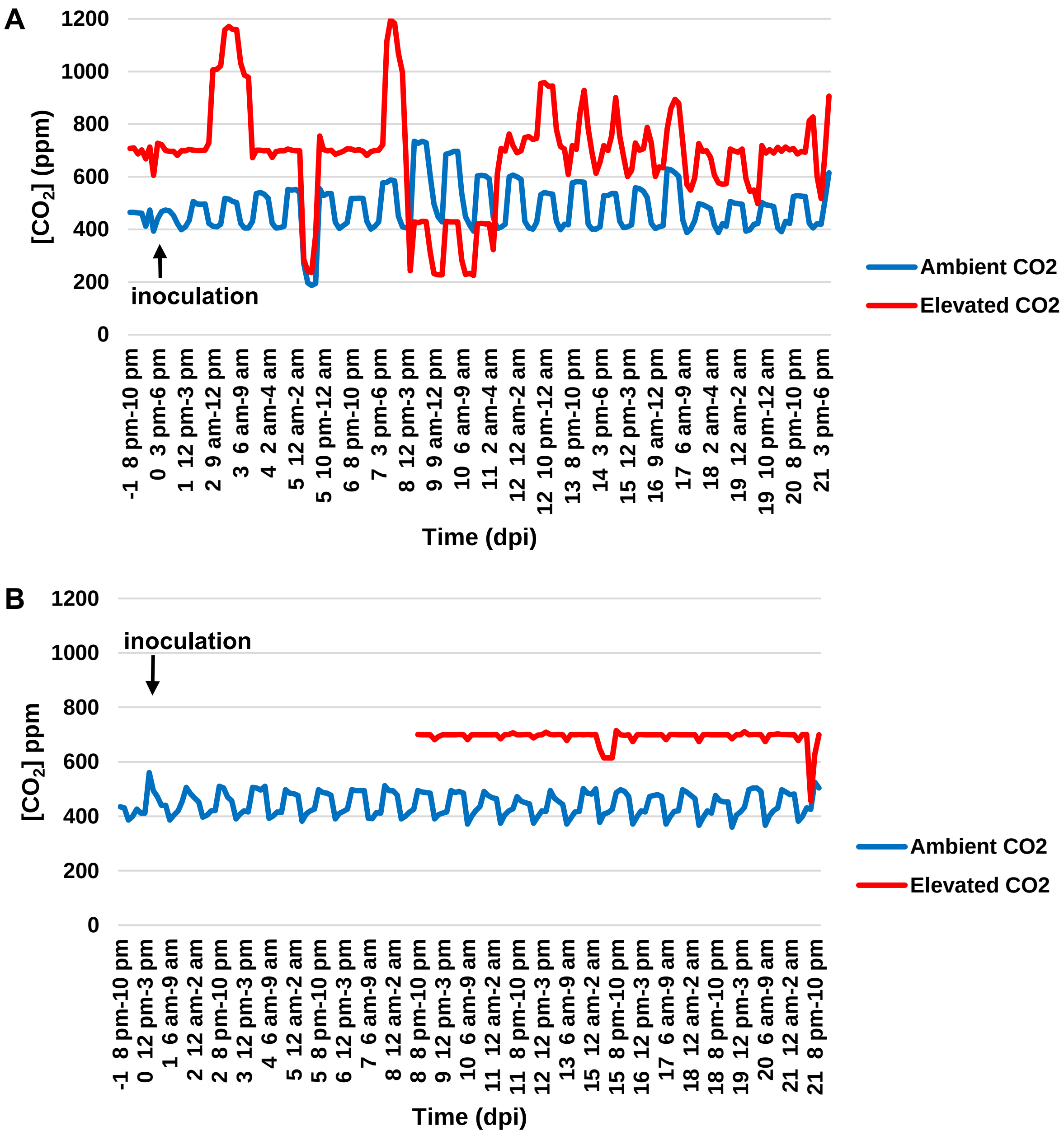

Figure 8. [CO_2] (ppm) in the ambient and elevated CO_2 growth chambers around the inoculation period (0-21 dpi) for trial 1 (A) and 2 (B). Eight median values were plotted per day. Data values are missing/erroneous for the elevated CO_2 chamber from 0-8 dpi for trial 2.

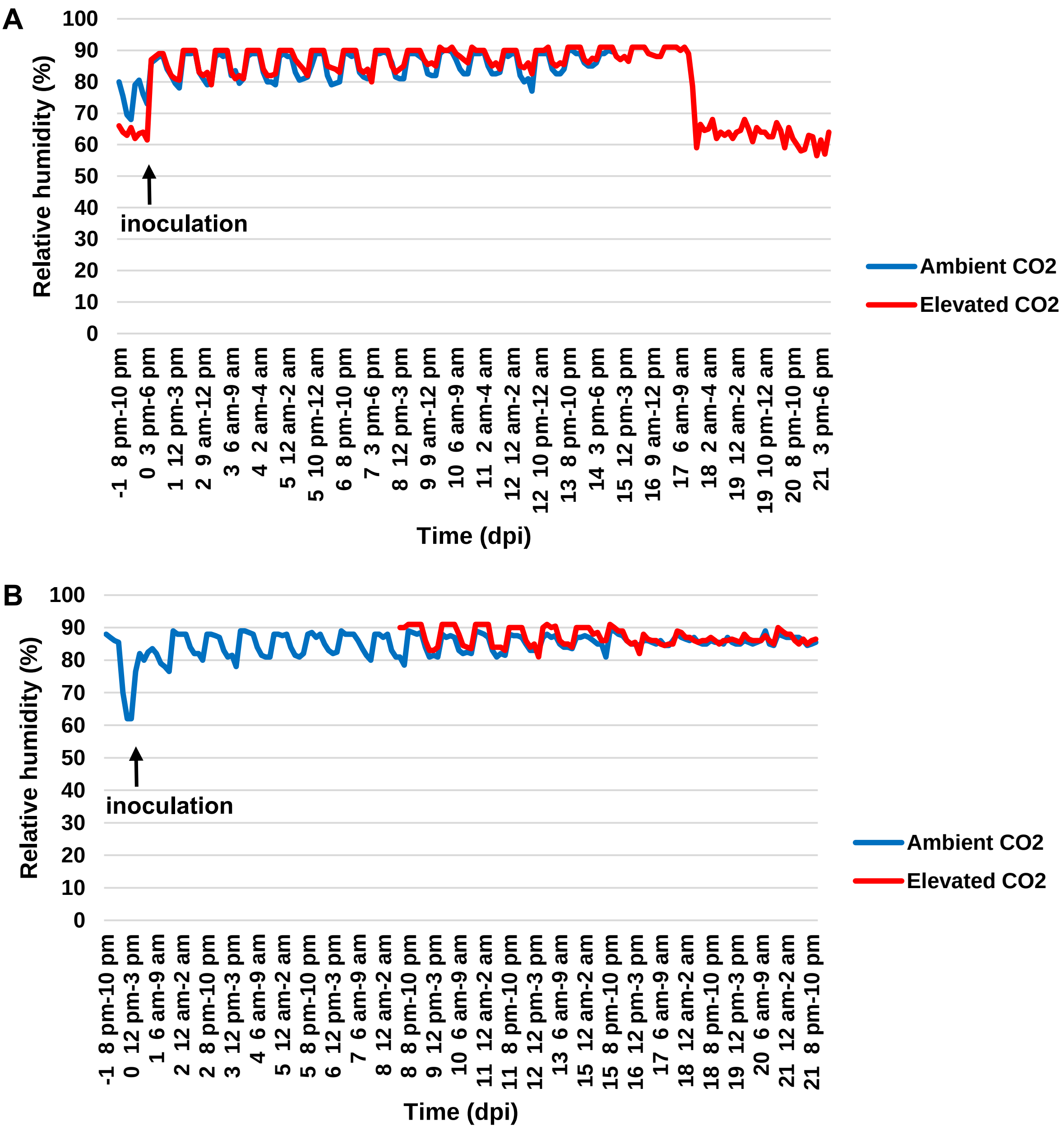

Figure 9. Relative humidity (%) in the ambient and elevated CO_2 growth chambers around the inoculation period (0-21 dpi) for trial 1 (A) and 2 (B). Eight median values were plotted per day. Data values are missing/erroneous for the ambient CO_2 chamber from 15-21 dpi for trial 1 and for the elevated CO_2 chamber from 0-8 dpi for trial 2.

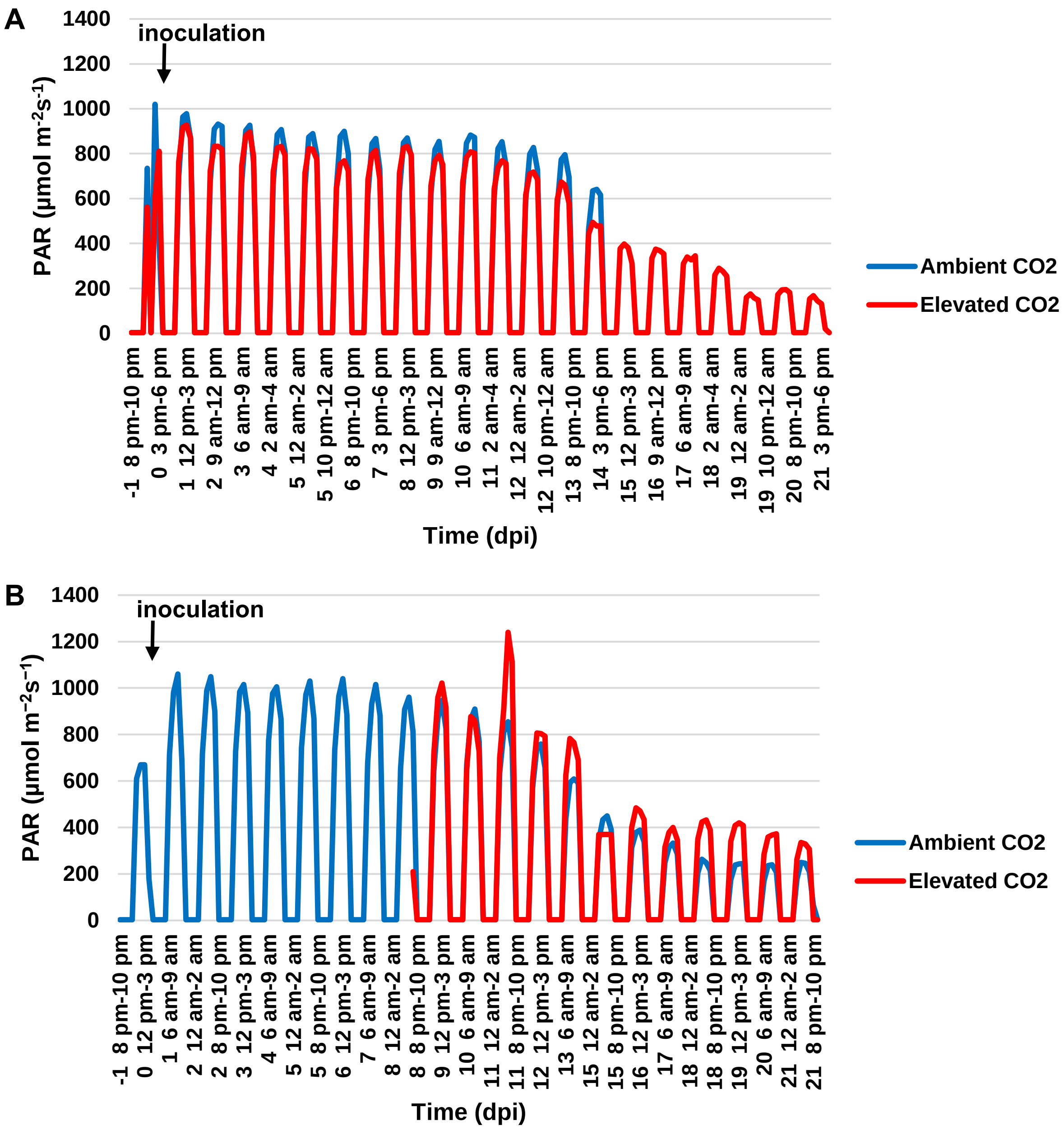

Figure 10. PAR intensity (μmol m^{-2}s^{-1}) in the ambient and elevated CO$_2$ growth chambers around the inoculation period (0-21 dpi) for trial 1 (A) and 2 (B). Eight median values were plotted per day. Data values are missing/erroneous for the ambient CO$_2$ chamber from 15-21 dpi for trial 1 and for the elevated CO$_2$ chamber from 0-8 dpi for trial 2.

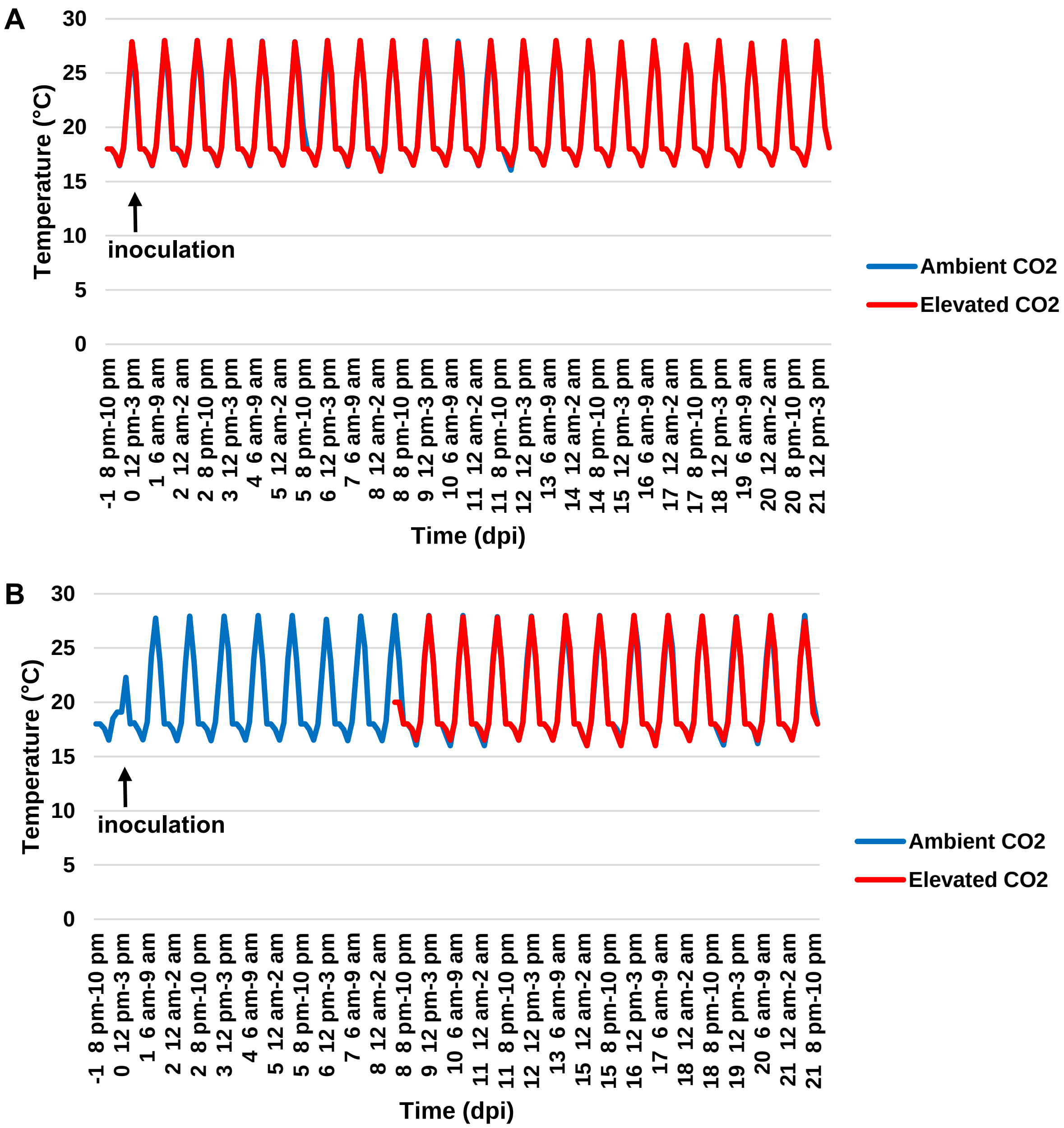

Figure 11. Temperature (°C) in the ambient and elevated CO_2 growth chambers around the inoculation period (0-21 dpi) for trial 1 (A) and 2 (B). Eight median values were plotted per day. Data values are missing/erroneous for the ambient CO_2 chamber from 15-21 dpi for trial 1 and for the elevated CO_2 chamber from 0-8 dpi for trial 2.

2.3.2.3 Gas exchange measurements

Net CO_2 assimilation (*A*) (photosynthetic rate) and stomatal conductance (g_{sw}) were measured using a LI-6800 portable photosynthesis machine. Maize leaf samples from each CO_2 treatment were assessed at four different time points (21, 28, 33 and 41 dpi) corresponding to four distinct stages of GLS symptom development (chlorotic spots, chlorotic lesions, single lesions and coalesced lesions) for both trials (Fig. S3 & S4). A significant difference in the photosynthetic rate (*A*) was observed between CO_2 treatments for the aggregate average of four time points for both trial 1 (Mann-Whitney *U* test, $P<0.01$) and trial 2 ($P<0.001$). The photosynthetic rate was 26.2% higher in the elevated CO_2-grown plants than ambient for trial 1 (Fig. 12A) and 36.3% greater for trial 2 (Fig. 12B). According to a Mann-Whitney *U* test, there was significantly higher (49.6%) photosynthetic rates in the mock-inoculated plants than the inoculated control maize leaves for trial 1 ($P<0.0001$) (Fig. 12C). This was also observed in trial 2 (Fig. 12D), although it was not a statistically significant difference ($P>0.05$). The younger leaves (V9) were significantly more photosynthetically active than the older leaves (V4, V5, V6), as greater *A* was documented with increasing leaf rank in each trial (1-way ANOVA with Bonferroni's multiple comparison test, $P<0.05$) (Fig. 12E & 12F).

The stomatal conductance values were averaged across four measurement dates (21, 28, 33 and 41 dpi). A significant difference (Mann-Whitney *U* test, $P<0.001$) in stomatal conductance was observed between CO_2 regimes, where 25.3% greater g_{sw} was recorded at elevated CO_2 in comparison with the ambient CO_2 maize leaves for trial 1 (Fig. 13A). However, this result was not replicated in the second trial, where [CO_2] had no significant effect (Mann-Whitney *U* test, $P>0.05$) on stomatal conductance (Fig. 13B). There was a statistically significant difference in stomatal conductance between inoculated and mock-inoculated maize leaves for trial 1 ($P<0.001$) (Fig. 13C), but not for trial 2 ($P>0.05$) (Fig. 13D), according to a Mann-Whitney *U* test. For trial 1, 44.8% lower stomatal conductance was measured for *C. zeina* infected leaf samples versus the control maize leaves, whereas the corresponding difference was 9% in trial 2. Stomatal conductance was positively correlated with leaf rank in both trials, such that the younger leaves facilitated significantly higher g_{sw} than the older leaves (1-way ANOVA with Bonferroni's multiple comparison test, $P<0.05$) (Fig. 13E & 13F).

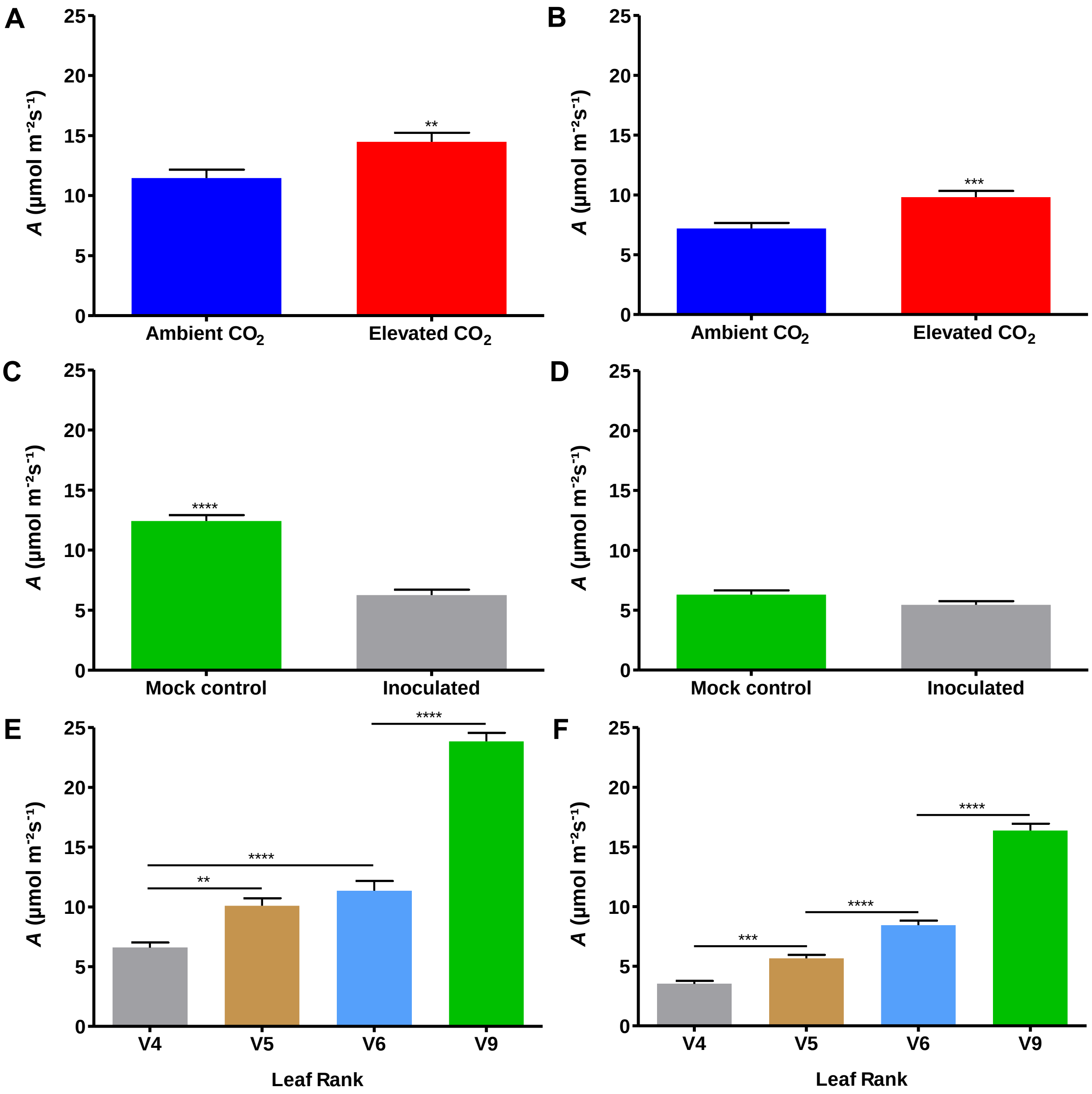

Figure 12. Net CO_2 assimilation rate (*A*) of maize leaves. A significant difference in the photosynthetic rate (*n* = 32, ±SEM) was observed between ambient and elevated CO_2 treatments for both trial 1 (***P*<0.01) (A) and trial 2 (****P*<0.001) (B), based on a Mann-Whitney *U* test. There was a significant difference in *A* (*n* = 32, ±SEM) between inoculated and mock-inoculated control maize leaves for trial 1 (*****P*<0.0001) (C), but not for trial 2 (D), based on a Mann-Whitney *U* test. Higher photosynthesis (*n* = 64, ±SEM) was positively correlated with leaf rank (1-way ANOVA with Bonferroni's multiple comparison test, ***P*<0.01; ****P*<0.001; *****P*<0.0001) for trial 1 (E) and trial 2 (F) (alpha = 0.05). Average values across 21, 28, 33 and 41 dpi samples are shown.

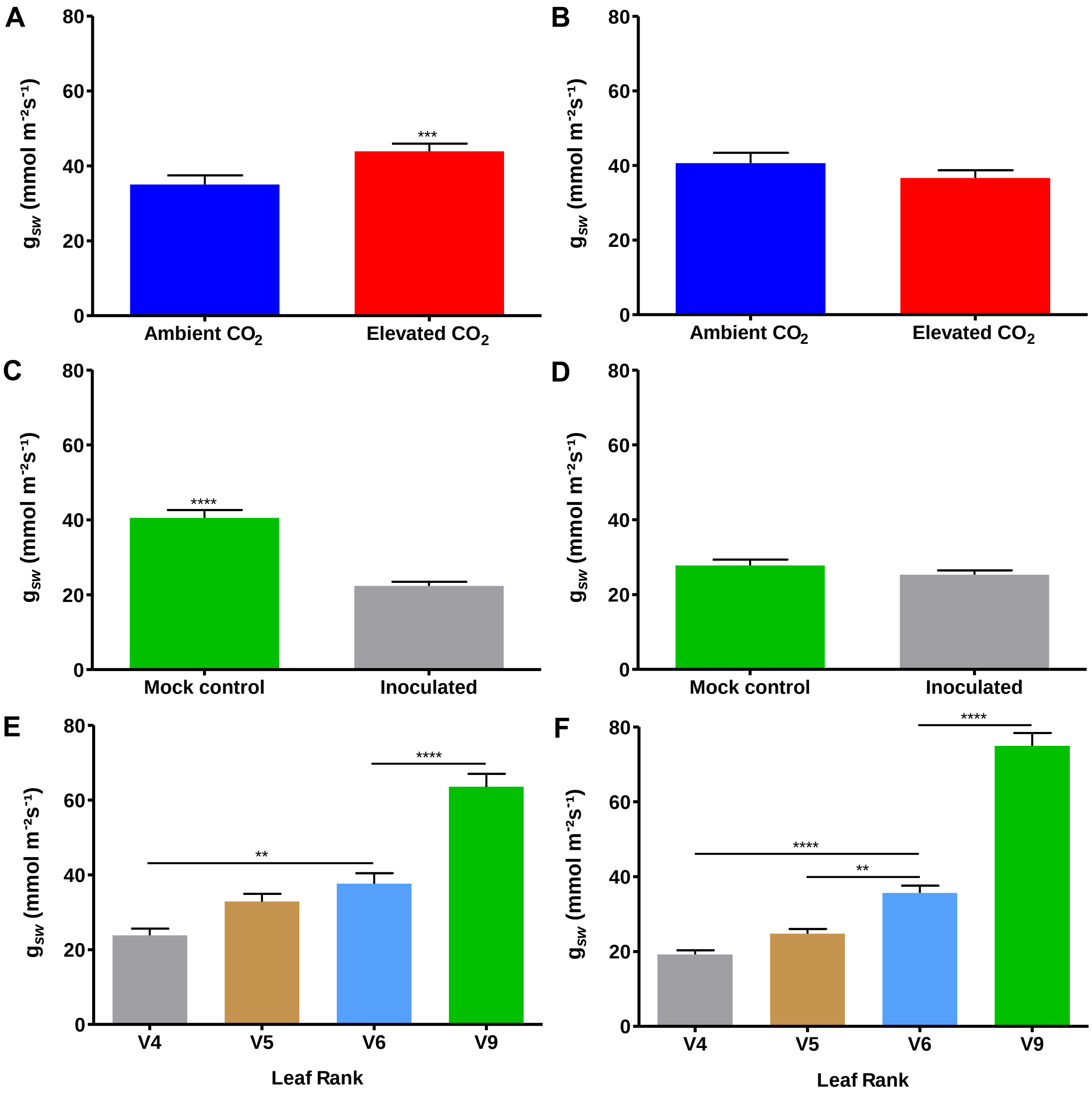

Figure 13. Stomatal conductance (g_{sw}) of maize leaves. A significant difference (***$P<0.001$) in stomatal conductance ($n = 32$, ±SEM) was observed between ambient and elevated CO$_2$ treatments for trial 1 (A), while no significant difference was reported for trial 2 (B), based on a Mann-Whitney *U* test. There was a significant difference in g_{sw} ($n = 32$, ±SEM) between inoculated and mock-inoculated control leaves for trial 1 (***$P<0.001$) (C), but not for trial 2 (D), based on a Mann-Whitney *U* test. Higher stomatal conductance ($n = 64$, ±SEM) was positively correlated with leaf rank (1-way ANOVA with Bonferroni's multiple comparison test, **$P<0.01$; ****$P<0.0001$) for trial 1 (E) and trial 2 (F) (alpha = 0.05). Average values across 21, 28, 33 and 41 dpi samples are shown.

2.3.2.4 Stomatal density counts

The stomatal density (SD) was determined by viewing both the adaxial and abaxial sides of maize leaf epidermal peels under a light microscope. Micrographs were taken from four biological replicates on the three ranks of inoculated/mock-inoculated leaves (bottom – V4, middle – V5, top – V6), as well as the most recent fully expanded leaf (new – V9), per plant at 65 dap (Fig. 14). Characteristic dumbbell-shaped stomata, composed of two guard cells, were observed running parallel to leaf venation, along with the surrounding epidermal cells.

Stomatal numbers were counted manually, and the SD calculated. A 56.7% higher stomatal density was recorded on the lower leaf surface in comparison with the upper surface, based on a Mann-Whitney *U* test ($P<0.0001$) (Fig. 15A). No significant difference (Mann-Whitney *U* test, $P>0.05$) in SD was observed between the ambient and elevated CO_2 treatments, (Fig. 15B). Furthermore, there was no significant difference (1-way ANOVA with Bonferroni's multiple comparison test, $P>0.05$) in stomatal density across all four leaf ranks sampled (bottom, middle, top and new) (Fig. 15C).

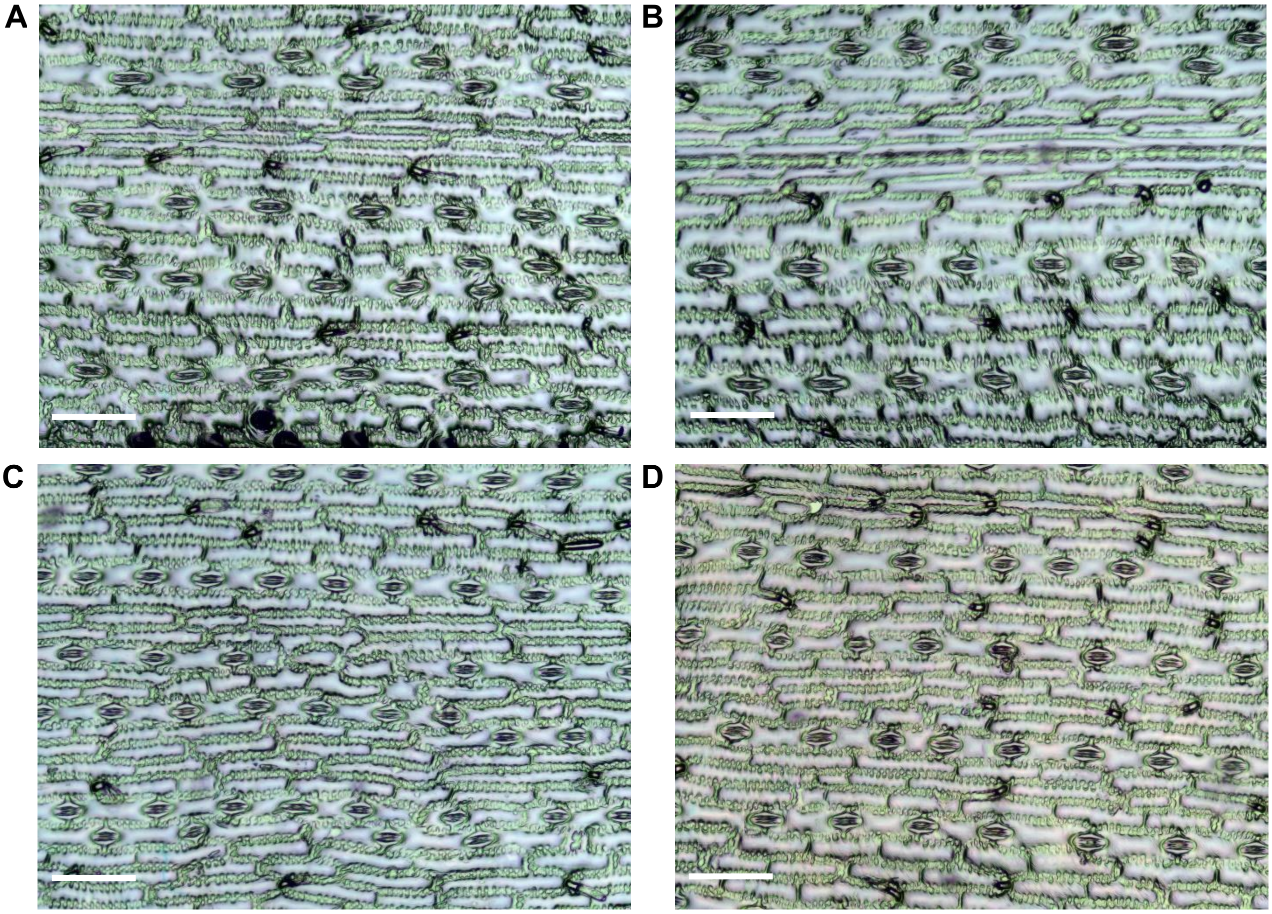

Figure 14. Micrographs of maize leaf stomata from the middle leaf rank at 100X magnification. (A) Adaxial surface at ambient CO_2. (B) Adaxial surface at elevated CO_2. (C) Abaxial surface at ambient CO_2. (D) Abaxial surface at elevated CO_2. Middle rank micrographs are representative of all the leaf ranks. Scale bar = 100 µm.

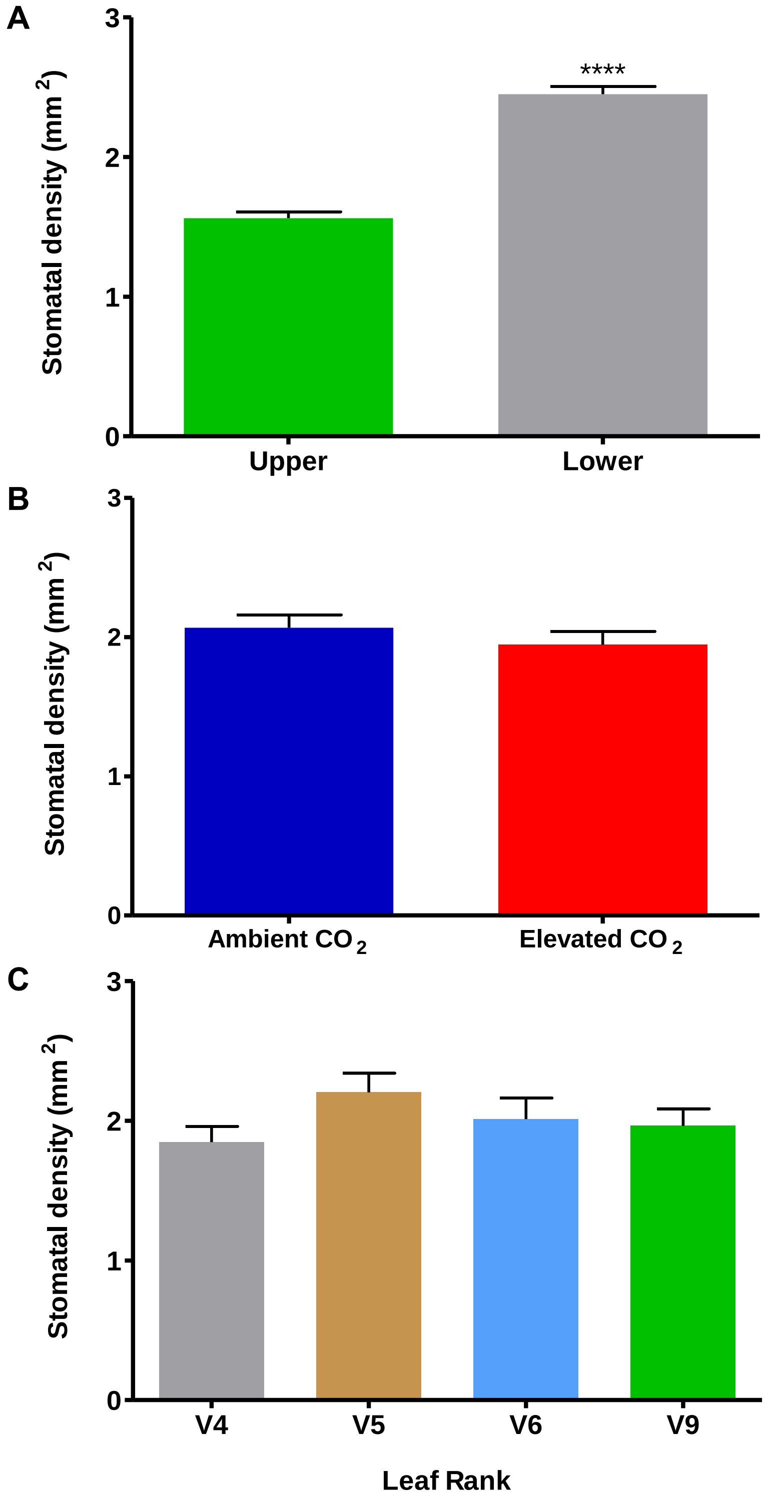

Figure 15. Stomatal density of maize leaves at 65 dap. A significant difference (*****P*<0.0001) in stomatal density (*n* = 32, ±SEM) was observed between the upper and lower leaf surfaces (A), while no significant difference was documented between the respective CO_2 regimes (B), based on a Mann-Whitney *U* test. (C) Stomatal density (*n* = 16, ±SEM) was relatively similar across leaf ranks, according to a 1-way ANOVA with Bonferroni's multiple comparison test (alpha = 0.05).

2.3.2.5 Metabolite analysis

Sugar assays were conducted on glucose, fructose, sucrose, maltose and trehalose to determine their respective carbohydrate levels in maize plants grown in the two CO_2 environments (Fig. S5). No significant differences (2-way ANOVA with Bonferroni's multiple comparison test, $P>0.05$) in glucose, fructose, sucrose and trehalose concentrations were observed between the ambient and elevated CO_2 treatments during trial 1. On the other hand, maltose levels were 42.5% greater at 415 ppm than 700 ppm CO_2 ($P<0.01$) (Fig. 16A). Metabolite concentrations were similar between inoculated and mock-inoculated maize leaves (Fig. 16B), based on a 2-way ANOVA with Bonferroni's multiple comparison test, $P>0.05$).

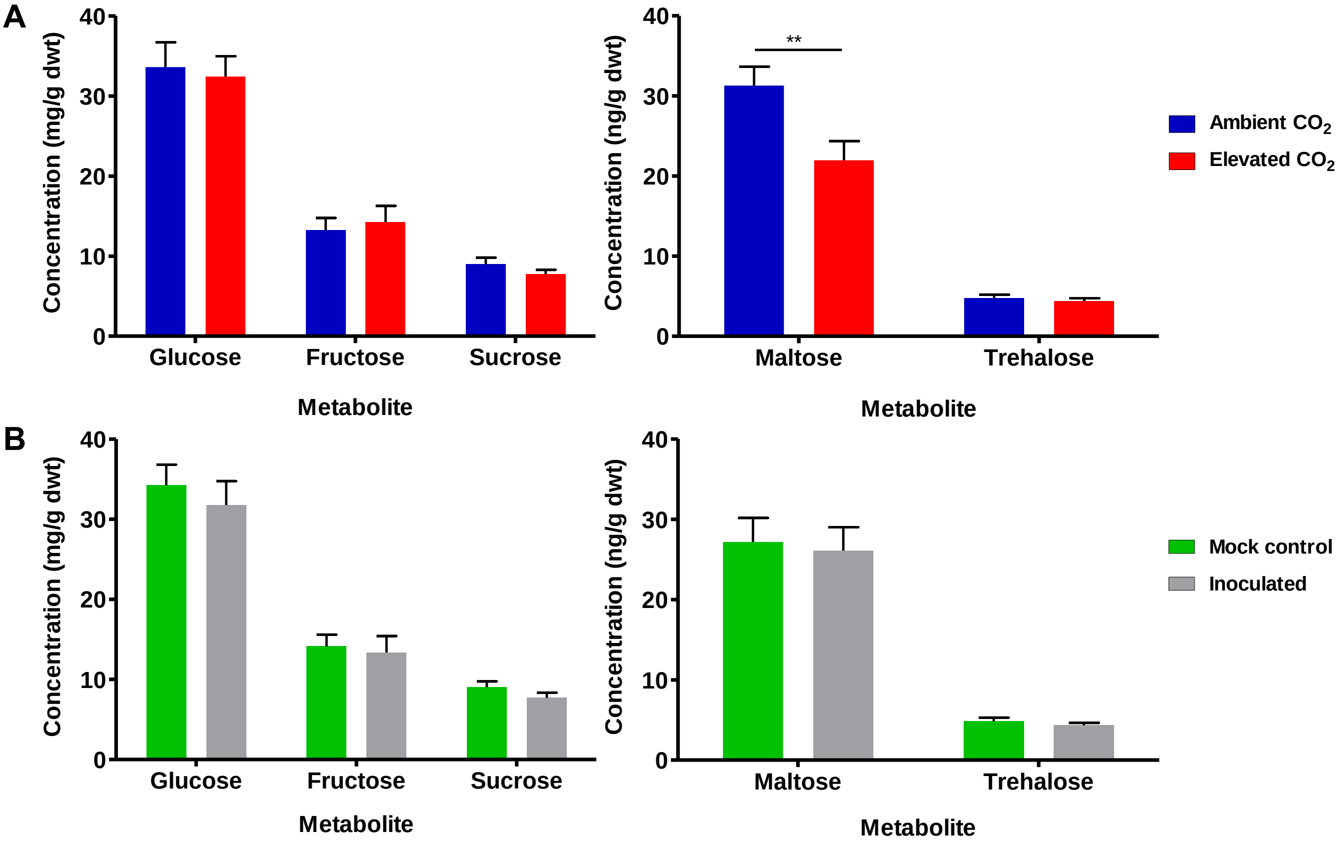

Figure 16. Maize leaf glucose, fructose, sucrose (mg/g dwt), maltose and trehalose (ng/g dwt) concentrations at ambient vs elevated [CO_2]. No significant difference in metabolite concentrations ($n = 40$, ±SEM) were observed between the respective CO_2 regimes for glucose, fructose, sucrose and trehalose, while there was a significant difference (**$P<0.01$) for maltose (A), according to a 2-way ANOVA with Bonferroni's multiple comparison test. No significant difference in metabolite levels ($n = 40$, ±SEM) were documented between the inoculated and mock-inoculated control leaves for all sugars (B), according to a 2-way ANOVA with Bonferroni's multiple comparison test (alpha = 0.05). Dwt, dry weight.

2.3.2.6 *In vitro C. zeina* growth

C. zeina strain Mkushi was grown *in vitro* as single colonies on V8 media at 415 and 700 ppm [CO_2] (Fig. 17 & 18). The experiment was repeated, with the first test comprising of 750 colonies/plate and the second 250 colonies/plate. Photos of the plates were taken approximately every three days and characteristic grey colonies were observed at 10 dpi for both experiments. By 18 dpi, the colonies in both experiments were marginally larger than before, but equally divided between light grey and black specks for experiment 1 and predominantly grey spots for experiment 2. The majority of colonies reached the vegetative growth phase and were white in colour, along with an expanded colony size at 23 dpi in both experiments. However, there was no significant difference (Mann-Whitney *U* test, $P>0.05$) in colony size (mm^2) between the two CO_2 treatments at any time point (Fig. 19). Additionally, colony morphology remained unaffected by the CO_2 levels in both experiments.

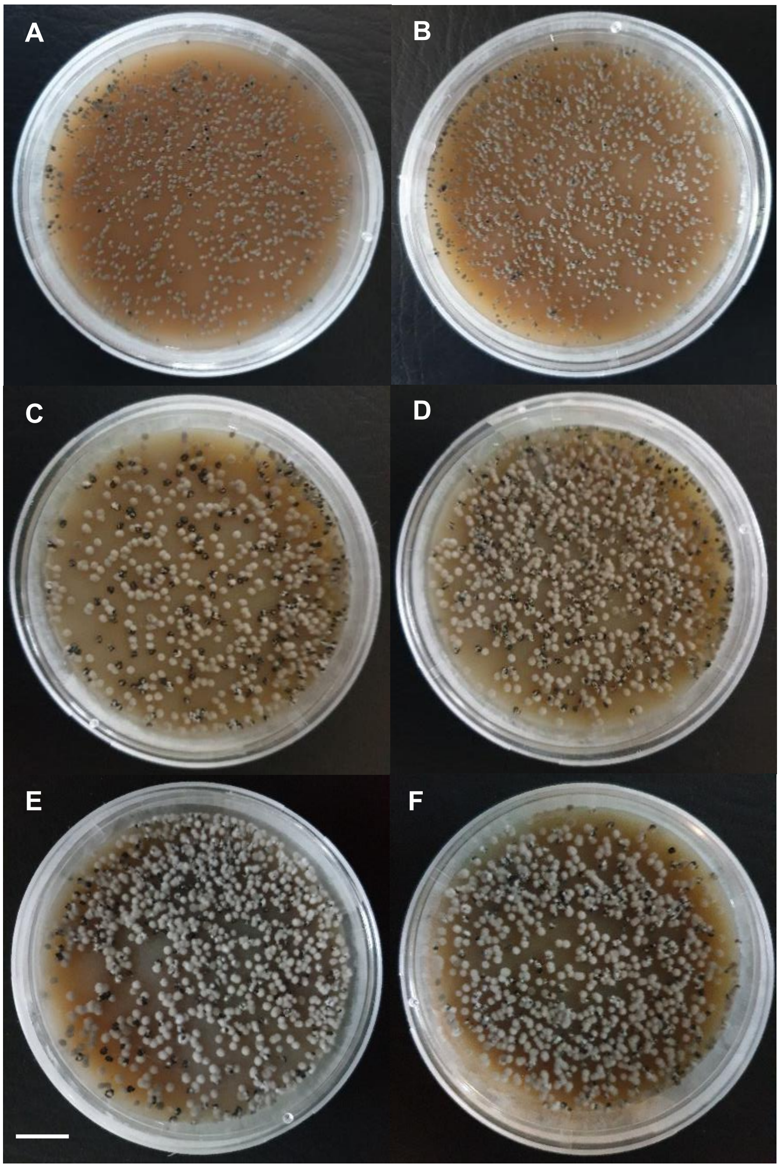

Figure 17. Representative photographs of *C. zeina* single colonies (750/plate) grown on V8 media at ambient (A, C, E) and elevated CO_2 levels (B, D, F). (A, B) 10 dpi; (C, D) 18 dpi; (E, F) 23 dpi. Scale bar = 1 cm.

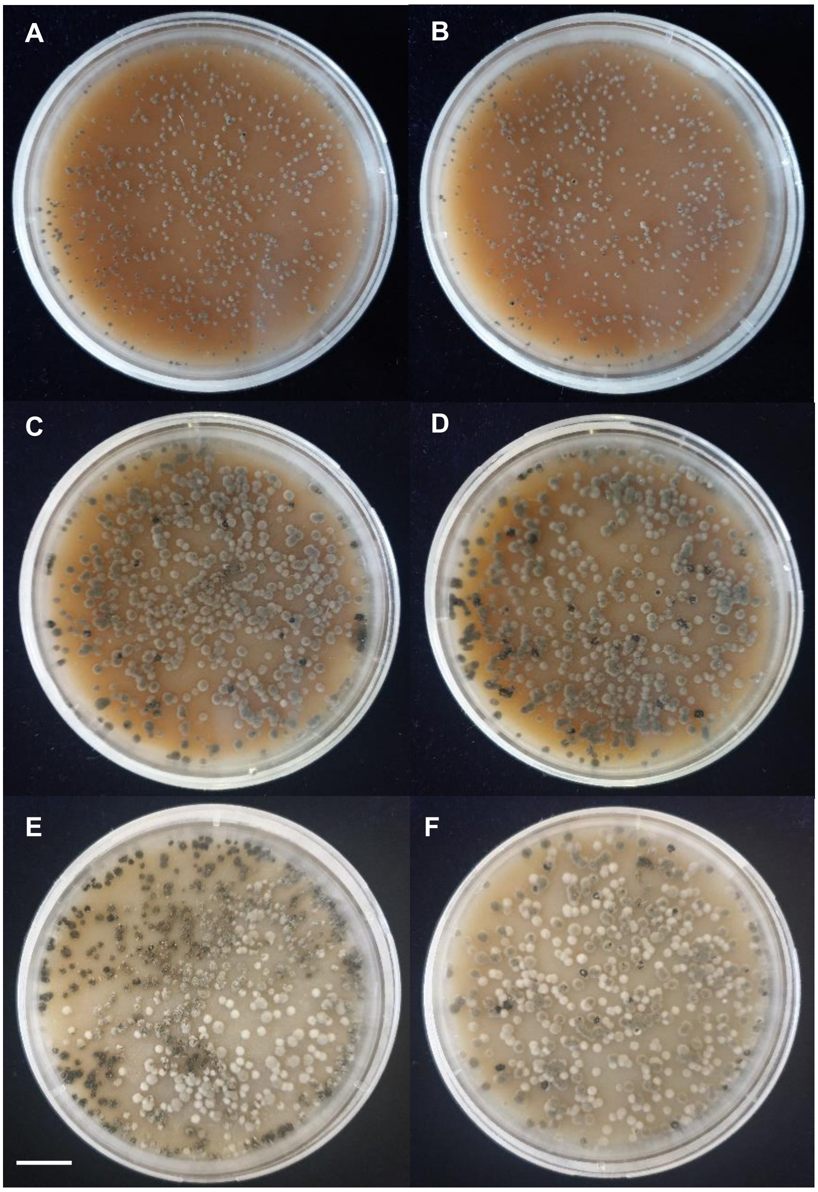

Figure 18. Representative photographs of *C. zeina* single colonies (250/plate) grown on V8 media at ambient (A, C, E) and elevated CO_2 levels (B, D, F). (A, B) 10 dpi; (C, D) 18 dpi; (E, F) 23 dpi. Scale bar = 1 cm.

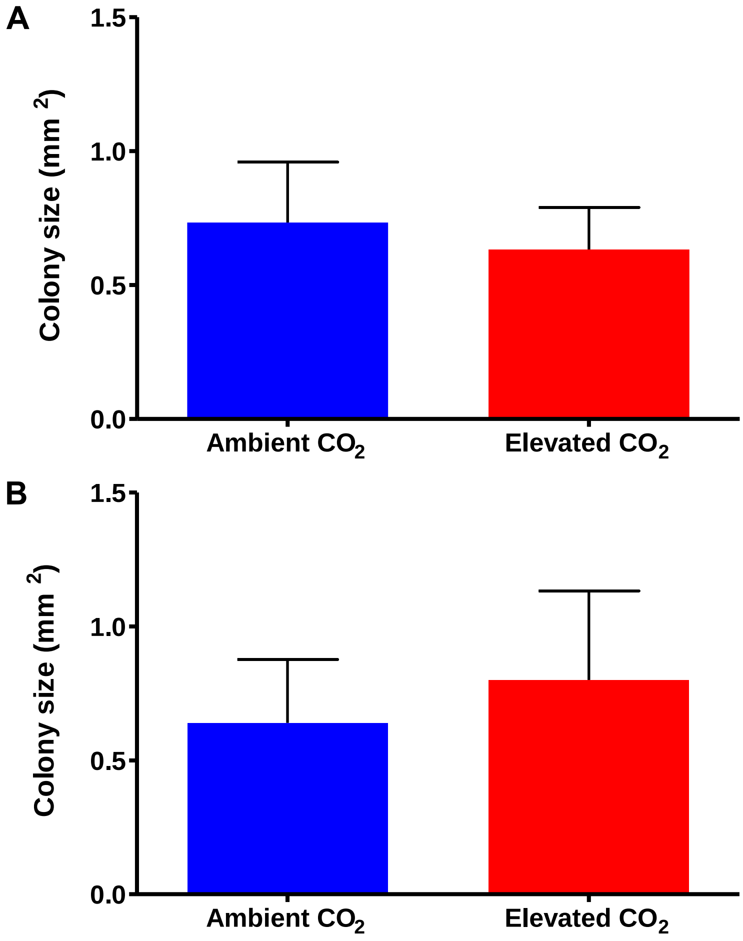

Figure 19. *In vitro C. zeina* colony growth at ambient and elevated CO_2 levels averaged over 23 days. (A) Experiment 1 consisted of 750 colonies/plate. (B) Experiment 2 consisted of 250 colonies/plate. No significant difference in colony size (*n* = 15, ±SEM) was reported between the CO_2 treatments for both experiments, based on a Mann-Whitney *U* test (alpha = 0.05).

2.3.2.7 GLS disease development

GLS disease development was monitored and recorded from the day of inoculation until 60 dpi by taking photographs of the 15 cm long inoculated/mock-inoculated zone. Disease scoring was done visually according to the S1-S6 scale (Table 4). In trial 1, initial symptoms in the form of yellow chlorotic spots were first observed at 21 dpi in both chambers. The yellow spots elongated to chlorotic lesions by 28 dpi for 75% of the inoculated leaves at ambient CO_2 and half of the samples at elevated CO_2. Several (9-15) single tan, rectangular lesions were seen at 33 dpi in the majority of ambient CO_2 plants, while their elevated CO_2 counterparts were evenly divided between a few (2-8) and several single matchstick lesions at the same time point. By 39 dpi, half of the ambient CO_2 maize exhibited several single lesions and the other half developed coalesced lesions (Fig. 20A). In the high CO_2 chamber at 39 dpi, 75% of the inoculated samples were at the single lesions stage, while coalesced lesions were present on the remaining maize leaves (Fig. 20B). Mature, coalesced lesions were observed on all leaf samples in both CO_2 regimes at 59 dpi. None of the mock-inoculated controls developed any GLS symptoms in either chamber for the duration of the experiment. Despite the minor differences in the timing of symptom progression, there was no statistically significant difference ($P>0.05$) in the overall development of GLS disease between the two CO_2 regimes, according to a two-way ANOVA with Bonferroni multiple comparison test (Fig. 21A).

Greater GLS disease incidence and severity were observed in the high CO_2 plants (Fig. 20D), as compared with the ambient CO_2 maize in trial 2 (Fig. 20C). Yellow chlorotic spots first developed on 30% of the inoculated leaves in the elevated CO_2 chamber and 10% of the ambient CO_2 maize at 14 dpi. At 21 dpi, the elevated CO_2 samples were divided between the chlorotic spots (30%) and chlorotic lesions (20%) disease stages and symptom-free maize (50%). On the other hand, 90% of the ambient CO_2 samples did not display any symptoms, while the remaining leaves had chlorotic lesions at 21 dpi. Only 30% of the plants at ambient CO_2 developed a few (2-8) single lesions at 33 dpi, as most of the maize leaves were symptomless. In contrast, half of the high CO_2 samples exhibited chlorotic symptoms and the other half single lesions (2-15). In the elevated CO_2 Conviron at 41 dpi, 30% of the inoculated samples were at the few single lesions stage, 40% at the several single lesions stage and 30% at the coalesced lesions stage. There were no symptoms on 70% of the ambient CO_2 maize and single lesions (20% few, 10% several) on the other sample leaves from 41-55 dpi. Mature coalesced lesions were present on 40% of the high CO_2 maize at 55 dpi, while the rest of the plant leaves displayed single lesions (30% few, 30% several). All mock-inoculated controls showed no GLS symptoms in both Convirons throughout the trial. A significant difference (two-way ANOVA with Bonferroni multiple comparison test, $P<0.05$) in disease scores were observed between the respective CO_2 treatments for the repeat trial, with enhanced GLS symptoms occurring at elevated [CO_2] versus ambient (Fig. 21B).

The area under the disease progress curve (AUDPC) scores directly corresponded to the disease scores, as no significant difference in GLS development was observed between CO_2 treatments for trial 1, whereas a significant difference ($P<0.05$) was reported for trial 2, based on a Mann-Whitney U test (Fig. S2).

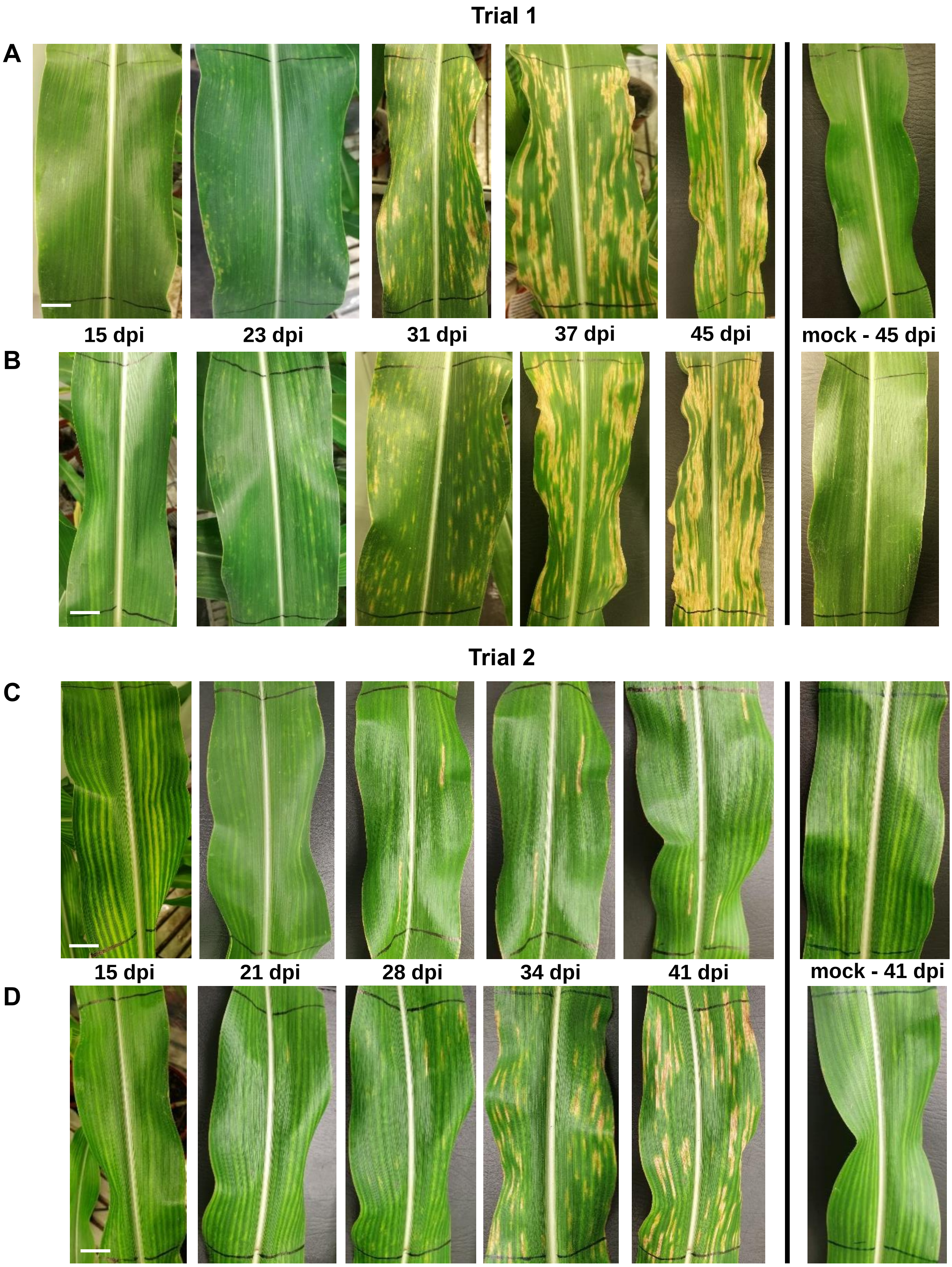

Figure 20. Representative photographs of GLS symptom development across five time points for inoculated (first five photos per row) and mock-inoculated (last photo per row) maize samples at ambient vs elevated [CO_2]. (A) Trial 1 ambient CO_2 samples. (B) Trial 1 elevated CO_2 samples. (C) Trial 2 ambient CO_2 samples. (D) Trial 2 elevated CO_2 samples. Scale bar = 2 cm.

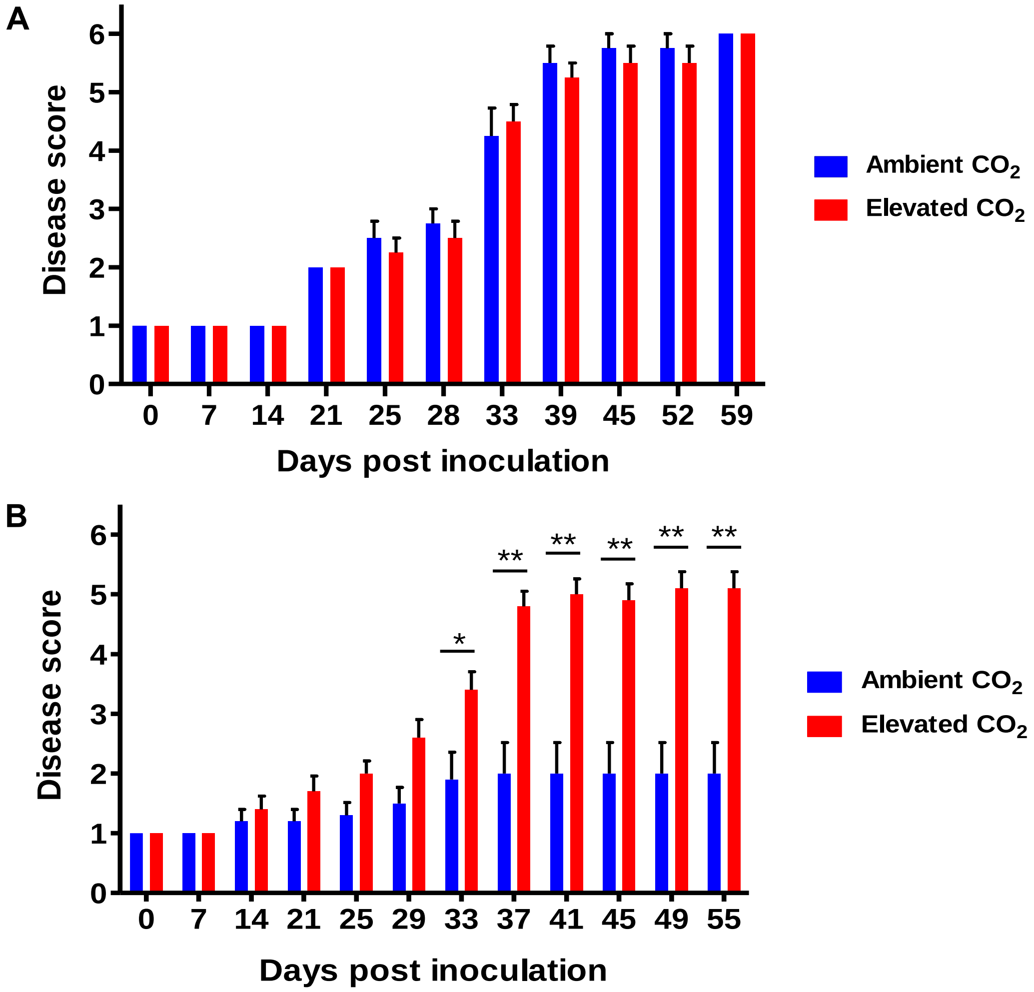

Figure 21. Disease scores for *C. zeina*-inoculated maize plants at ambient vs elevated [CO_2]. No significant difference in GLS symptom development (*n* = 10, ±SEM) was observed between CO_2 treatments for each respective time point for trial 1 (A) and a significant difference between CO_2 regimes from 33 dpi onwards for trial 2 (**P*<0.05, ***P*<0.0001) (B), based on a 2-way ANOVA with Bonferroni multiple comparison test (alpha = 0.05).

2.3.2.8 *In planta C. zeina* quantification

A qPCR assay was performed to quantify the *in planta* fungal DNA content between the respective CO_2 treatments. Genomic DNA was first extracted from the harvested 23 dpi (trial 2 only), 30 dpi and 44 dpi *C. zeina*-inoculated and mock-inoculated leaf samples following the CTAB protocol. NanoDrop readings indicated that the gDNA was of good quality and relatively pure, with mean concentrations of approximately 110 ng/µl and 260/280 ratios of ~1.64 for both trials. The integrity of the gDNA was validated, as solid high molecular weight bands were observed on a 1% agarose gel for all the samples analysed (Fig. 22A).

PCR amplification showed that single 106 bp *gst3* and 164 bp *cpr1_2* gene fragments were amplified for the respective primer sets (Fig. 22B & 22C). The samples corresponded to the following three distinct stages of GLS symptom development: chlorotic spots (23 dpi), single lesions (30 dpi) and coalesced lesions progressing to severe blight (44 dpi), as well as the symptomless mock controls.

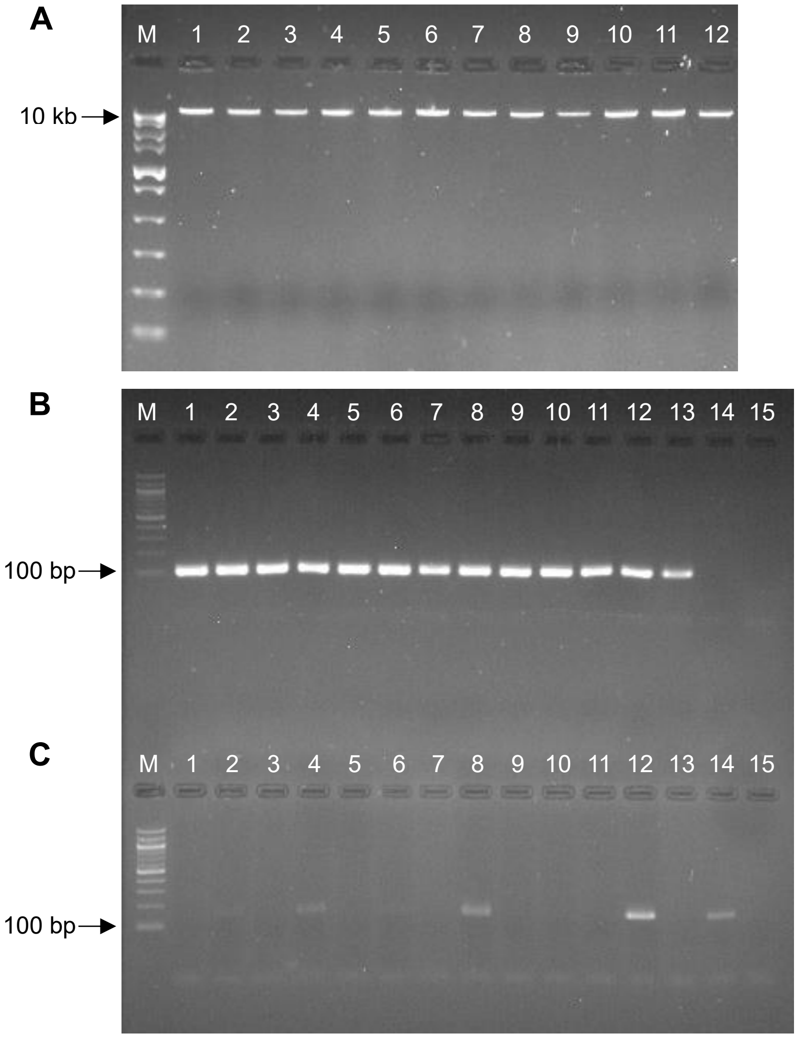

Figure 22. (A) 1% agarose gel showing high molecular weight gDNA extraction fragments from inoculated and mock-inoculated maize samples. Lanes 1-4, 23 dpi; lanes 5-8, 30 dpi; 9-12, 44 dpi. Lane M, Fast DNA ladder (NEB); lanes 1, 5 and 9, ambient CO_2 mock; lanes 2, 6 and 10, ambient CO_2 inoculated; lanes 3, 7 and 11, elevated CO_2 mock; lanes 4, 8 12, elevated CO_2 inoculated. (B) PCR amplification of 106 bp *gst3* gene fragments from inoculated and mock-inoculated maize samples. Lanes 1-4, 23 dpi; lanes 5-8, 30 dpi; 9-12, 44 dpi. Lane M, 100 bp (NEB); lanes 1, 5 and 9, ambient CO_2 mock; lanes 2, 6 and 10, ambient CO_2 inoculated; lanes 3, 7 and 11, elevated CO_2 mock; lanes 4, 8 12, elevated CO_2 inoculated; lane 13, positive control (B73 maize gDNA); lane 14, negative control (*C. zeina* gDNA); lane 15, no-template control. 1.4% agarose gel. (C) PCR amplification of 164 bp *cpr1_2* gene fragments from inoculated and mock-inoculated maize samples. Lanes 1-4, 23 dpi; lanes 5-8, 30 dpi; 9-12, 44 dpi. Lane M, 100 bp (NEB); lanes 1, 5 and 9, ambient CO_2 mock; lanes 2, 6 and 10, ambient CO_2 inoculated; lanes 3, 7 and 11, elevated CO_2 mock; lanes 4, 8 12, elevated CO_2 inoculated; lane 13, negative control (B73 maize gDNA); lane 14, positive control (*C. zeina* gDNA); lane 15, no-template control. 1.4% agarose gel. Gels are representative of both trials.

Prior to conducting the qPCR assay, a standard curve was prepared across 6-dilution points for the *gst3* primers and 8-dilution points for the *cpr1_2* primers. A qPCR efficiency of 92.8% was achieved for the *gst3* primers and 100.1% for the *cpr1_2* primers, which are both within the desired range for standard curve slopes. The correlation coefficient (R^2) was 0.96 for the *gst3* standard curve and 0.99 for the *cpr1_2* curve, which indicates that the assay was optimised (Fig. 23). Melt curve analysis indicated that a single product was amplified for each well for both sets of primers (Fig. 24), which was validated by gel electrophoresis. Gene quantification was normalised between the different samples according to the delta-delta Ct ($2^{-(\Delta\Delta Ct)}$) method by dividing the amount of fungal *cpr1_2* by the amount of maize *gst3* quantified in infected leaves (ng *C. zeina* DNA/mg maize DNA).

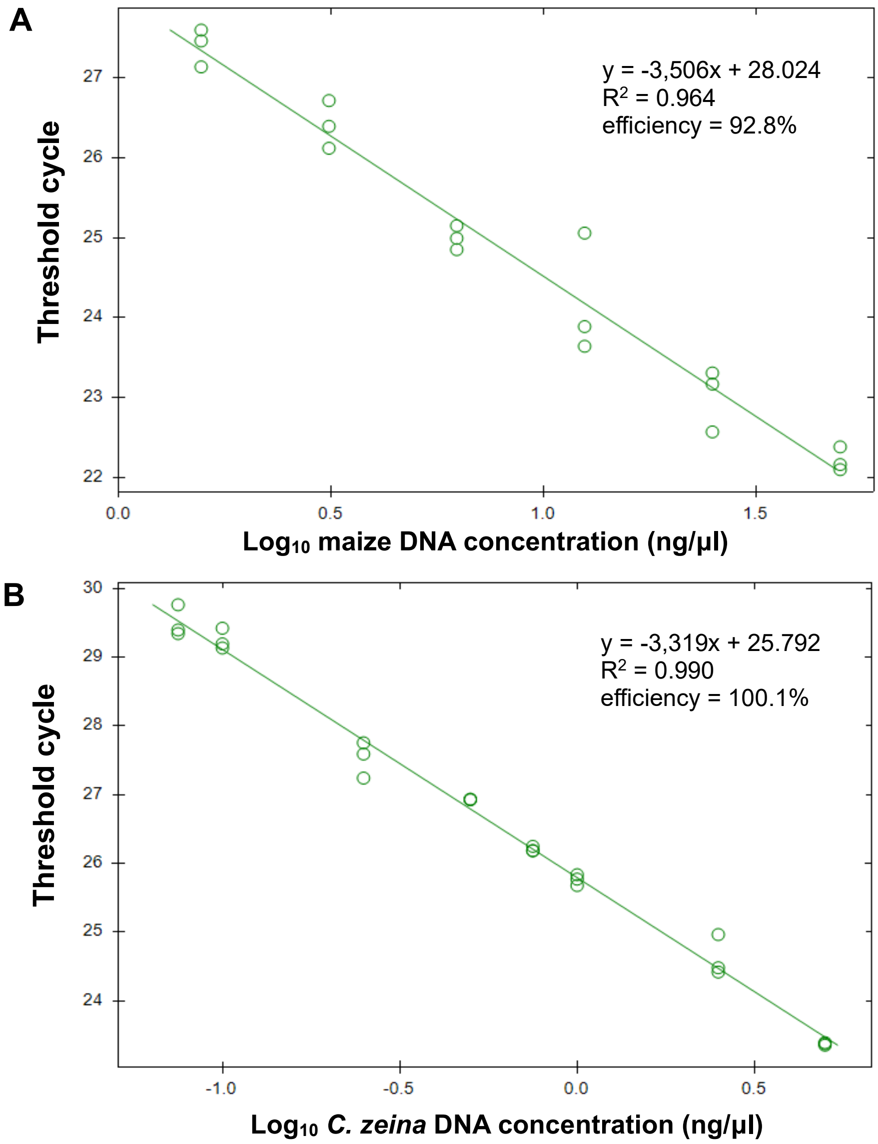

Figure 23. Standard curves for *in planta* assay indicating qPCR efficiency. (A) *gst3* amplification from a 6-point serial dilution of maize gDNA. (B) *cpr1_2* amplification from an 8-point serial dilution of *C. zeina* gDNA in carrier maize gDNA. Three technical replicates are shown for each dilution point. Standard curves are representative of both trials.

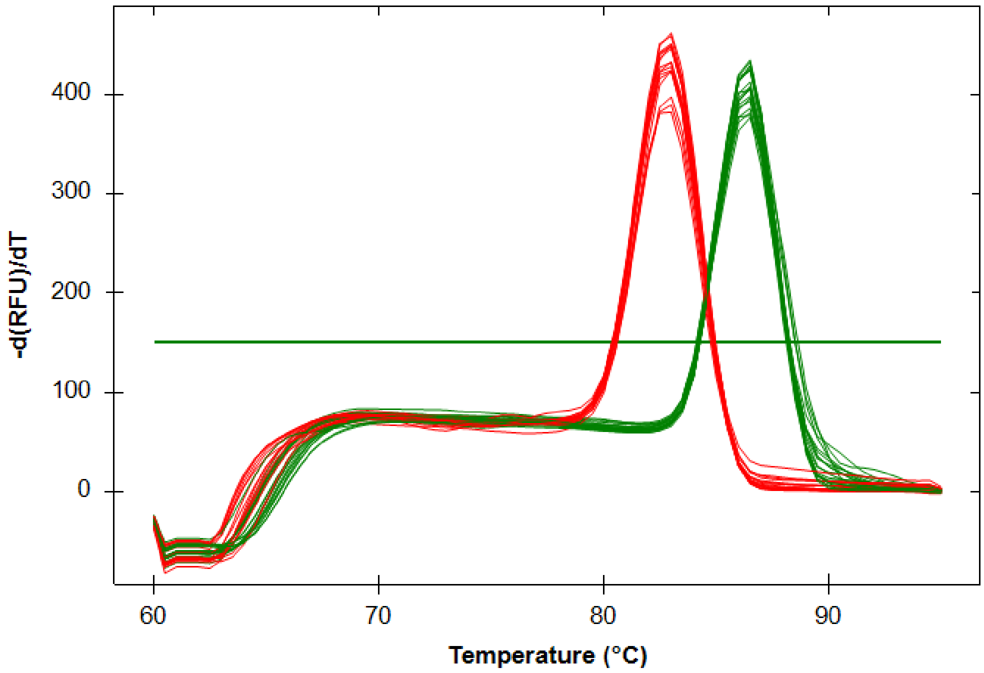

Figure 24. Melting temperature (Tm) graphs for the *gst3* amplicon (red) from maize gDNA and *cpr1_2* amplicon (green) from *C. zeina* gDNA. Graphs for multiple biological and technical replicates of each primer pair are shown and representative of both trials.

In planta quantification of *C. zeina* was conducted using a qPCR assay designed specifically for GLS disease on maize leaves. Maize leaf fungal content was averaged across two stages of symptom development sampled: chlorotic spots and single lesions. No significant difference in fungal content was documented between the respective CO_2 treatments for trial 1 (Mann-Whitney *U* test, $P>0.05$), despite quantifying 1.5-fold greater *C. zeina* content in the elevated CO_2 maize in comparison with their ambient CO_2 counterparts. On the other hand, a significant difference was reported for trial 2 ($P<0.05$), according to a Mann-Whitney *U* test, where 19-fold higher fungal content was recorded in the elevated CO_2 maize than the ambient CO_2 plants (Fig. 25).

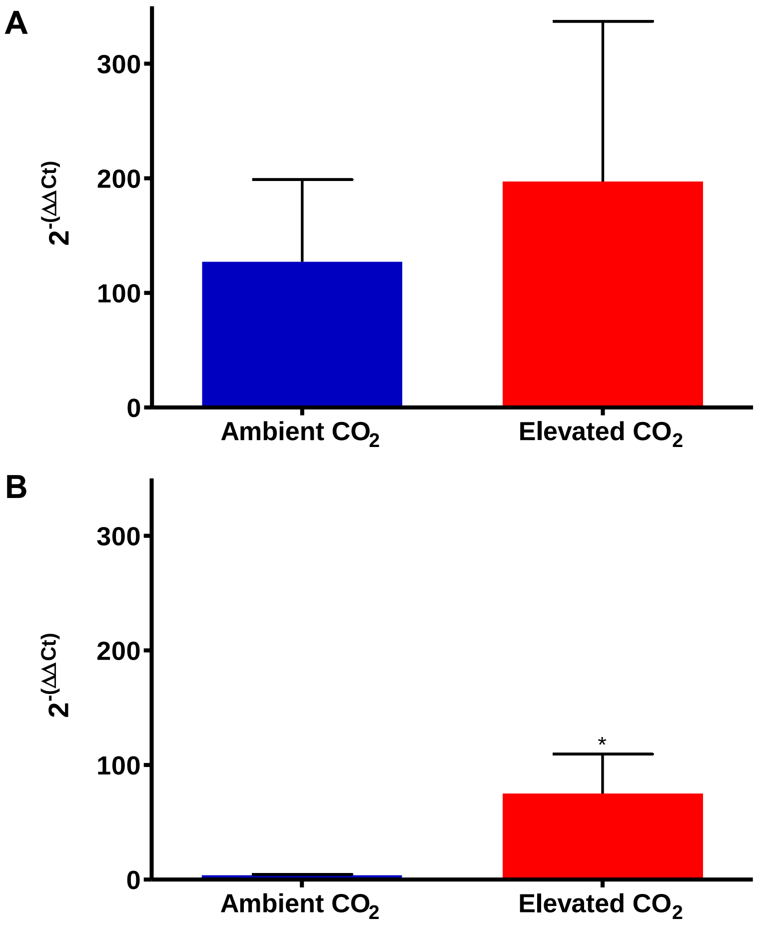

Figure 25. qPCR assay to quantify the *in planta C. zeina* content from inoculated maize samples at ambient vs elevated [CO_2]. The delta-delta Ct ($2^{-(\Delta\Delta Ct)}$) method was used to calculate relative quantification. Gene quantification was normalised by dividing the amount of *cpr1_2* by the amount of *gst3* quantified in infected leaves. No significant difference in fungal content ($n = 10$, ±SEM) was documented between CO_2 treatments for trial 1 (A) and a significant difference was reported for trial 2 (**P*<0.05) (B), based on a Mann-Whitney *U* test (alpha = 0.05). Average values across 30 and 44 dpi samples are shown.

2.4 Discussion

Research has demonstrated the multitude of ways elevated CO_2 concentrations affect maize plants and fungal pathogens, however the intersection point of a maize disease, such as GLS, remains a global concern (Coakley et al., 1999, Kazan, 2018, Ainsworth and Long, 2021). In this study, the effect of CO_2 enrichment on maize susceptibility to grey leaf spot (GLS) was examined in a chamber disease trial over two growing seasons. No significant difference in maize plant height, leaf stomatal density and *in vitro C. zeina* growth was observed between the ambient and elevated CO_2 regimes in all experiments. The photosynthetic rate (*A*) and stomatal conductance (g_{sw}) were assessed, and greater photosynthetic rates were detected for B73 plants grown at 700 ppm CO_2 in both trials, whereas higher stomatal conductance was measured on the elevated CO_2-grown plants in trial 2 only. GLS disease resulted in a reduction in both leaf photosynthetic rates and stomatal conductance, which were positively correlated with leaf rank. Maize leaf disease development was not significantly affected by CO_2 enrichment in trial 1 and comparable GLS symptom formation was documented for the elevated CO_2-grown maize leaves in the repeat trial. However, GLS disease incidence and severity were very low in the ambient CO_2 Conviron in trial 2. Chamber environmental data point towards a chamber effect occurring in trial 2, whereby the ambient CO_2 chamber achieved a relatively lower RHmax following inoculation. Low relative humidity is unfavourable for *C. zeina* infection, so the observed low GLS disease incidence for the ambient CO_2 treatment is thus likely a false response due to the malfunctioning growth chamber. It is therefore concluded that maize susceptibility to GLS disease will be unaffected by elevated [CO_2], in accord with the trial 1 observations.

An extensive range of research has demonstrated that CO_2 enrichment will benefit C_3 plant species in terms of increased growth and yields (Ehleringer and Björkman, 1977, Miri et al., 2012). Selected studies on maize and other C_4 species have reported that elevated [CO_2] can result in enhanced growth, despite the CO_2 concentrating mechanism associated with the C_4 photosynthesis system (Wong, 1979, Maroco et al., 1999). However, a number of more recent studies, including meta-analyses, have shown that maize growth is not affected by high CO_2 levels in the absence of drought stress (Leakey et al., 2006, Manderscheid et al., 2014, Ainsworth and Long, 2021). In both trials of this study, no significant difference in maize growth (height) was recorded between 415 and 700 ppm CO_2 treatments under well-watered conditions, even though CO_2 assimilation rates (*A*) were higher for the elevated CO_2 treatments. This result was in accordance with another chamber study, where maize growth was shown to be unaffected by elevated [CO_2] of 650-800 ppm versus ambient (Hunt et al., 1991). Similarly, Prins et al. (2011) grew maize plants at 350 and 700 ppm CO_2 and reported no change in growth in an enclosed chamber trial under well-watered conditions. The results observed are also consistent with a number of FACE trials that have demonstrated identical maize growth rates at ambient (376-390 ppm) and elevated CO_2 (550-585 ppm) (Leakey et al., 2006, Manderscheid et al., 2014, Ruiz-Vera et al., 2015). Therefore, the weight of evidence shows that maize growth will not be influenced by high CO_2 conditions.

Photosynthesis is a means of converting radiant energy into useable carbohydrates and is thus a measure of leaf productivity, as well as being functionally correlated with the process of gas exchange between the plant and the environment (Sage, 1994). Elevated CO_2 levels were observed to have a significant effect on the photosynthetic rate (*A*) of maize leaves in this investigation, as *A* was 26.2% and 36.3% greater in the high CO_2 plants versus ambient for trial 1 and trial 2, respectively. The results documented in this study are consistent with some of the established literature, as 1.2-fold heightened photosynthesis was reported at elevated [CO_2] of 640 ppm in comparison with the ambient CO_2 (330 ppm) maize in a glasshouse trial (Wong, 1979). The same effect was seen at even greater CO_2 levels (1100 ppm) in a chamber study, where photosynthetic rates were 15% higher than in the ambient leaves (Maroco et al., 1999). The enhanced photosynthesis at 700 ppm versus 415 ppm CO_2 observed in this study indicates that while plant growth (height) is not affected, other maize physiological parameters can be influenced by a CO_2 enriched environment. On the other hand, a few chamber trials have reported no significant difference in photosynthesis between maize cultivated at 350 ppm and 700 ppm CO_2 (Driscoll et al., 2006, Prins et al., 2011). FACE experiments have also documented analogous *A* between ambient (376-400 ppm CO_2) and elevated CO_2 (550-800 ppm) treatments in maize (Leakey et al., 2006, Vaughan et al., 2014). These contrasting outcomes may be due to the influence of other abiotic factors and variable environmental conditions. The different maize varieties and methods of measurement taking used in the various studies also generates inconsistency and further research is required.

The hypothesized effect of GLS reducing the photosynthetic capacity of maize leaves was confirmed in this study, as it was shown that infected leaves had lower rates of photosynthesis than the control samples. The effect was only significant in the first trial, however, where a 49.6% decrease was noted. The chlorotic and necrotic lesions, as well as the leaf blighting formed by proliferating *C. zeina*, degrade the photosynthetic leaf tissue and diminish kernel development (Ward et al., 1999). Furthermore, increasing *A* was recorded with rising leaf rank in both trials, since the younger maize leaves were significantly more photosynthetically active than the older leaves. Thus, leaf productivity is a highly variable across the maize plant and may increase due to CO_2 enrichment.

Stomatal conductance is an indication of the uptake of CO_2 and loss of water vapour through leaf stomata and is positively correlated with stomatal opening, since it is a function of the stomata's parameters (size and density) (Xu et al., 2016). In trial 1 of this study, increased g_{sw} (25.3%) was observed in the elevated CO_2 leaves in comparison with the ambient CO_2 maize. However, decreased stomatal conductance at 700 ppm CO_2 was documented for the repeat trial, although it was not statistically significant. The trial 2 result is in accordance with findings in comparable studies, such as the enclosed chamber trial conducted by Maroco et al. (1999), who quantified a 71% decrease in stomatal conductance in maize grown at 1100 ppm CO_2 in comparison with the ambient CO_2 plants. Similarly, Prins et al. (2011) reported decreased g_{sw} in maize leaves at 700 ppm CO_2 versus the plant leaves grown at 350 ppm CO_2 in a chamber study. Therefore, the trial 1 readings are likely inaccurate due to fluctuating chamber conditions, specifically at the time of taking the measurements. This is supported by the Conviron environmental data, which indicates that there were [CO_2] spikes (~500-600 ppm) in the ambient chamber at 21, 28, 33 and 44 dpi and a dip (~300-400 ppm CO_2) in concentration in the elevated CO_2 chamber at 41 dpi that could affect leaf gas exchange and thus modify g_{sw} readings. The CO_2 levels were maintained relatively well in both Convirons during the course of the second trial and few notable spikes or dips occurred on the days when gas exchange measurements were recorded. Additionally, sample leaves with GLS symptoms had lower rates of stomatal conductance than the uninfected maize, although it was only a significant difference in the first trial, where a 44.8% reduction was documented. This demonstrates that GLS infection is associated with enhanced stomatal closure, which may be a defence response by the maize leaf, as pathogen-associated molecular patterns (PAMPs), such as fungal toxins and cell wall components, can promote stomatal closure (Lake and Wade, 2009). Stomatal conductance was also shown to be positively correlated with maize leaf rank in both trials, as the younger leaves had significantly greater g_{sw} than the older leaves. Hence, stomatal conductance is adaptable and reflective of the ever-changing dynamics between the maize leaf and the surrounding environment.

Stomatal density (SD) is the number of stomata per unit area and can be influenced by the [CO_2], granting that this effect is more prominent in C_3 species than C_4 plants (Zhao et al., 2015). Maize leaves are amphistomatous, since they have stomata on both the adaxial and abaxial surfaces, although they are more abundant on the abaxial side (Juarez et al., 2004). This was confirmed in this study, as 56.7% greater SD was observed on the abaxial leaf surface than the adaxial side. Additionally, the results from this study indicate that stomatal density was not affected by elevated CO_2 levels (700 ppm), which is consistent with the observations of Driscoll et al. (2006), who reported that SD was unchanged between the maize grown at 350 ppm and double ambient CO_2. Similarly, elevated [CO_2] of 550 ppm were shown to have no effect on stomatal density in comparison to ambient levels (350 ppm CO_2) in one FACE study (McElrone et al., 2005). Stomatal density was relatively comparable across the different leaf ranks corresponding to the V4, V5, V6 and V9 leaf stages. CO_2 has been demonstrated to function in signal transduction processes that impact leaf development and plants can thus control stomatal density by transmitting information concerning CO_2 concentration from older to younger leaves (Lake et al., 2001, Hu et al., 2010). The difference in [CO_2] between treatments or the leaf rank range may have not been sufficient to produce a significant change in SD, while this phenomenon may simply not occur in B73 maize. *C. zeina* enters through maize leaf stomata during GLS infection, so any changes in the number of stomata could affect fungal load and disease severity. Unchanging SD is thus consistent with the comparable GLS disease development observed between CO_2 treatments in trial 1. Therefore, although stomatal density can be a plastic trait, it was not affected by CO_2 enrichment under the conditions of this study.

Maize sugars play roles in gene expression, host defence, signalling, as well as regulating various cellular responses (Sheen, 1994, Bolouri Moghaddam and Van den Ende, 2012). An assortment of primary maize metabolites, including glucose, fructose and sucrose, have been examined at elevated [CO_2]. In this study, glucose, fructose, sucrose and trehalose concentrations were observed to be unaffected by elevated CO_2 levels, whereas maltose concentrations were 42.5% higher at 415 ppm than 700 ppm CO_2. In a related FACE experiment, no significant difference in total leaf carbohydrate content, as well as glucose, fructose and sucrose levels, were documented between the 376 ppm- and 550 ppm CO_2-grown maize plants (Leakey et al., 2006). Sicher and Barnaby (2012) analysed maize leaf metabolites in a chamber study and found that high CO_2 treatment (~700 ppm) did not affect the concentration of fructose, glucose or sucrose when compared with the ambient CO_2 samples. The reason for the relatively low maltose levels in the elevated CO_2 environment is unclear and requires further experimentation, however, there is evidence that light influences maltose concentrations (Sharkey et al., 2004, Lu et al., 2005). Radiation levels were marginally greater in the ambient CO_2 Conviron than the elevated CO_2 chamber for trial 1, which may account for the observed differences. Sugars function in co-regulating plant defences and enhanced concentrations of carbohydrates can induce host resistance during pathogen infection by supplying additional energy resources needed for downstream responses (Gómez-Ariza et al., 2007, Bolouri Moghaddam and Van den Ende, 2012). However, metabolite analysis of the inoculated and mock-inoculated maize leaves in trial 1 showed that glucose, fructose, sucrose, trehalose and maltose levels were not influenced by GLS infection. The future climate is thus predicted to produce negligible effects on the levels of several maize leaf sugars, but more research is required into other metabolites.

Few studies have examined the *in vitro* growth of pathogenic fungi at elevated CO_2 levels, while there have been a few reports on fungal *in planta* growth that can also provide means of comparison. *C. zeina* (Ascomycota) radial growth was not influenced by CO_2 enrichment in this investigation, as was observed in two single colony *in vitro* experiments. Similarly, researchers documented that there was no significant difference in the *in planta* growth of two distinct subspecies of *Fusarium oxysporum* (ascomycete pathogen) between ambient (400 ppm CO_2) and double ambient treatments (Ferrocino et al., 2013, Chitarra et al., 2015). A CO_2-rich environment was shown to have no effect on the *in vitro* growth of the oomycete *Phytophthora parasitica* when grown on V8C agar at 350 and 700 ppm [CO_2] (Jwa and Walling, 2001). On the other hand, the fungal foliar pathogen *Phyllosticta minima* was cultured on potato dextrose agar (PDA) at 360 ppm and 560 ppm CO_2 in a growth chamber experiment, which reported a 17% greater *in vitro* growth rate at elevated CO_2 concentrations (McElrone et al., 2005). *Erysiphe graminis* is an ascomycete that causes powdery mildew on cereals and its *in planta* established colonies grew significantly faster and larger at elevated [CO_2] (700 ppm) in comparison with the colonies isolated from barley leaves grown at 350 ppm CO_2 (Hibberd et al., 1996b). Additionally, Vaughan et al. (2014) found that the *in planta* biomass of *Fusarium verticillioides* increased significantly in stalk-inoculated maize plants at CO_2 levels of 800 ppm versus ambient concentrations (400 ppm CO_2). *C. zeina* is an anamorphic fungus that grows as mycelia on V8 media and then reproduces asexually via conidial spores, although mating type (MAT) ratios indicate that it has a cryptic teleomorph stage (Nsibo et al., 2019). Colony morphology was identical between the V8 plates grown in the respective CO_2 conditions in both experiments. McElrone et al. (2005) did not observe any changes in *P. minima* colony morphology between ambient and elevated CO_2 regimes in a similar *in vitro* study. However, fungal pathogens, such as *Cryptococcus neoformans* and *Candida albicans*, have displayed altered morphology and development *in vitro* due to elevated CO_2 concentrations (Rocha et al., 2001, Alspaugh et al., 2002, Sheth et al., 2005). There is a dissimilarity between *in vitro* and *in planta* environments due to the volatile interactions that occur within host plants, but are absent in artificial conditions, so it remains possible that pathogens may respond differently in the respective settings. Therefore, differing outcomes are possible for fungal pathogens, but much more research is required in terms of assessing the response to CO_2 enrichment *in vitro* and *in planta.*

The disease trial was performed twice to generate two seasons of data, however, technical issues with the Conviron growth chambers resulted in inconsistencies between the trials. The chambers designated as ambient (ID 001) and elevated CO_2 (ID 002) could not be switched between trials due to the inability of Conviron 1 to maintain the required elevated concentration of 700 ppm CO_2. The exact nature and timing of GLS infection, in terms of the incubation and latent periods, remains poorly characterised, therefore pinpointing the precise time at which the conditions need to be conducive to *C. zeina* proliferation is extrapolative (Lyimo et al., 2013). Disease symptoms first emerged in both chambers at 21 dpi for trial 1 and 14 dpi for trial 2, which demonstrates that the latent period for GLS disease was not affected by elevated CO_2 levels. Chakraborty et al. (2000a) observed the same result when assessing the fungal pathogen *Colletotrichum gloeosporioides* (Ascomycota) that causes anthracnose at 700 ppm versus 350 ppm CO_2. Analysis of the Conviron environmental data (particularly RH), specifically the 21-day period from maize inoculation to initial GLS symptom formation, provided an account of the discrepancies in disease development in trial 2 in the context of what is known about *C. zeina* infection.

The alternate hypothesis (H_1) for this project was that less GLS disease development would occur on maize leaves at elevated [CO_2] than ambient concentrations. This was based on several studies that have documented a decrease in maize leaf stomatal conductance, which directly corresponds to greater stomatal closure (Maroco et al., 1999, Prins et al., 2011). A reduction in GLS disease could occur based on the infection method of the fungus, as *C. zeina* enters through the stomata and greater stomatal closure could decrease the amount of pathogen able to penetrate the leaf (Lyimo et al., 2013). A lowered fungal load could result in the reduced development and severity of GLS symptoms. The newly formed leaves and stems were continuously cut to prevent excessive shading, which in addition to the anthocyanin stress response that was observed on some plant parts, could elicit a general defence response by priming downstream reactions. However, the experimental results did not correspond with the H_1, as increased stomatal conductance at elevated [CO_2] and unchanged stomatal density were recorded between CO_2 regimes, along with analogous GLS disease development in the primary trial. The difference in disease development in trial 2 is likely not a CO_2 effect, as similar symptom progression was observed between CO_2 regimes during trial 1, despite the spikes and dips in [CO_2], especially around 0-21 dpi. The CO_2 levels were in accordance with the respective 415 and 700 ppm CO_2 set points and were relatively stable for most of trial 2, including the inoculation to initial symptom phase. It is also evident from the pilot study that elevated CO_2 is not a necessary requirement for GLS disease incidence and progression in the growth chambers. Hence, the contrasting disease scores reported for the repeat trial are inconsistent with a CO_2 effect and taken together with the other experimental observations leads to rejection of the alternate hypothesis. This equates to validation of the null hypothesis (H_0), which posits there to be no distinction in maize susceptibility to GLS disease between ambient and elevated [CO_2].

The other environmental conditions controlled by the Convirons, specifically the temperature and light (PAR), could also contribute to the results obtained. Field observations at high levels of initial inoculum show that substantial GLS symptoms can develop at a wide range of temperatures, although fewer lesions form when daily mean temperatures drop below 20°C (Ward et al., 1999). Mean daily temperatures were around 20°C for both trials, and temperature regulation was relatively stable across the trials from 0-21 dpi and is therefore unlikely to be responsible for the differential disease results. PAR levels could have played a role in the distinct outcomes, but there is no clear pattern in the environmental data that accounts for both trials. The inaccuracy of the PAR measurements due to leaf shading and sensor positioning further complicates any observations. The PAR readings were slightly greater in the ambient CO_2 chamber than the elevated CO_2 Conviron for trial 1 around inoculation and this did not result in a significant difference in GLS symptoms, therefore, a comparable difference in PAR intensity between the chambers for trial 2 would not be expected to cause the substantial difference that was recorded. It is also worth noting that the PAR in the smaller PGCFlex Convirons is lower (~275 µmol $m^{-2}s^{-1}$) than in the larger PGW40 chambers and significant disease development (much greater than the ambient plants for trial 2) still occurred during the pilot study. Thus, the evidence indicates that chamber temperature and light conditions did not significantly influence the outcomes of the disease trials.

In trial 1, the chamber dehumidifiers were functioning correctly, and the relative humidity was very similar between the ambient and elevated CO_2 chambers from 0-21 dpi. However, the dehumidifiers, particularly the unit in the high CO_2 Conviron (ID 002), became faulty during trial 2 and had to be turned off at 15 dap, which resulted in limited humidity control. Environmental data from the elevated CO_2 chamber during trial 2 suggests that this Conviron was more suitable for GLS symptom development by providing higher levels of humidity, which are known to be beneficial for disease development by promoting germination of *C. zeina* conidia (Ward et al., 1999). The ambient CO_2 chamber maintained a lower and less prolonged relative humidity than the elevated CO_2 chamber during trial 2, which correlated with correspondingly producing a lower *in planta* fungal content, according to the qPCR assay. Greater *in planta* fungal content was quantified in maize leaf samples from both chambers in trial 1 than in either chamber in trial 2. This provides a rationale for the significant difference in disease development between CO_2 regimes during the second trial being an artefact of poor GLS symptom development in the ambient CO_2 chamber. Furthermore, B73 is a susceptible maize variety and the particular *C. zeina* strain (Mkushi) used was demonstrably infectious, so the variable environmental conditions (RH) are the probable cause of the discrepancies documented.

The microclimate around maize leaves is another factor that can play a role in disease development (Chakraborty et al., 2000b), as the environment directly around the stomata can provide favourable zones for *C. zeina* spore germination. There were 38 maize plants in the ambient CO_2 chamber and 39 in the elevated CO_2 chamber for the first trial, while 35 plants were housed in both chambers for the second trial. Greater leaf crowding due to the number of plants within each chamber can enhance the creation of stomatal microclimates and vice versa, so the lower humidity in the ambient CO_2 chamber during trial 2, coupled with a fewer number of plants provided a less advantageous environment for GLS symptom development in comparison with the other chambers. Therefore, the trial 1 result is hypothesized to be correct and the inconsistencies in trial 2 due to a chamber effect, with humidity the most plausible causal candidate. The chamber effect is posited to be due to the variation in relative humidity, which is known to influence *C. zeina* proliferation and thus GLS disease development (Ward et al., 1999). Humid conditions have been documented to be particularly beneficial for foliar diseases, such as leaf spots, blights, powdery mildews and rusts (Coakley et al., 1999). The trial 1 results, which illustrated that elevated [CO_2] did not have a significant effect on GLS, are theorised to be correct and consistent with other experimental work. On the other hand, the distinction in the trial 2 results is due to the lower humidity achieved in the ambient CO_2 chamber during the course of this trial and particularly during the pivotal 21-day period from inoculation to initial symptom development.

Rising CO_2 concentrations could dynamically modify the disease triangle for many plant-pathogen relationships, since all three elements can be influenced by [CO_2]. The limited studies on plant-pathogen interactions in response to CO_2 enrichment has produced variable results in C_3 and C_4 species, with cases of increased, decreased and unchanged susceptibility and severity being reported (Garrett et al., 2006, Yáñez-López et al., 2012). GLS disease was concluded to be unaffected by elevated [CO_2] (700 ppm) in comparison to ambient conditions (415 ppm CO_2) in this investigation. Correspondingly, the severity of powdery mildew infection on C_3 winter wheat, which is caused by the ascomycete pathogen *Erysiphe graminis*, was identical between ambient (350 ppm) and double ambient CO_2 treatments (700 ppm) in a glasshouse trial (Thompson et al., 1993). A phytotron trial revealed that CO_2 enrichment (800 ppm) did not have a significant effect on Fusarium wilt (*F. oxysporum* f.sp. *lactucae*) severity of lettuce (C_3) in comparison with the ambient control (400 ppm CO_2) (Ferrocino et al., 2013). An analogous result was seen in *Phytophthora cajani* (oomycete) infection of pigeon pea (C_3), whereby Phytophthora blight incidence was comparable between 380 ppm, 550 ppm and 700 ppm CO_2 treatments in a chamber study (Sharma et al., 2015). In contrast, one open-top chamber study observed incidences of increased severity of fungal infection in the C_4 grass *Spartina patens* at 700 versus 350 ppm CO_2 (Thompson and Drake, 1994). Fusarium wilt (*F.*

oxysporum f.sp. *conglutinans*) disease incidence on the C_3-C_4 intermediate rocket was greater at elevated CO_2 levels (800 ppm) in comparison to ambient (400 ppm CO_2) in a phytotron trial (Chitarra et al., 2015). Similarly, Vaughan et al. (2014) found that high [CO_2] (800 ppm) caused enhanced maize susceptibility to *F. verticillioides* versus the ambient treatment (400 ppm CO_2), although fumonisin content was unaltered. Greater grey mould (*Botrytis cinerea*) symptoms were observed in C_3 tomato plants grown at 800 ppm than 380 ppm CO_2 in a chamber experiment (Zhang et al., 2015). CO_2 enrichment has been shown to attenuate maize defences, such as jasmonic acid (JA), lipoxygenases (LOXs) and phytoalexins (Vaughan et al., 2014). This would decrease disease resistance and as a result credible concerns remain for maize pathogens other than *C. zeina*, as well as for the assortment of C_3 and C_4 plant diseases.

Decreased severity and susceptibility has been documented in a few high CO_2 disease trials examining a variety of plant-pathogen interactions. In one chamber experiment, Hibberd et al. (1996b) found that truncated primary penetration, which occurred post leaf inoculation, led to a reduction in powdery mildew (*E. graminis*) of C_3 barley at 700 ppm CO_2 versus ambient. Similarly, the severity of *C. gloeosporioides*-induced anthracnose decreased in response to CO_2 enrichment (700 ppm) in susceptible *Stylosanthes scabra* plants (C_3) in a chamber trial. However, no CO_2 effect on disease development was seen in a moderately resistant cultivar, which illustrates the variability of outcomes within the same host species (Chakraborty et al., 2000a). Both the incidence (27%) and severity (50%) of foliar leaf spot disease, which is caused by *P. minima* in C_3 red maple trees, were lower at 550 ppm than 350 ppm CO_2 in an open field experiment. The researchers attributed this effect to a reduction in host nutritive quality and altered leaf chemistry (McElrone et al., 2005). Zhang et al. (2015) reported that elevated [CO_2] (800 ppm) resulted in diminished susceptibility to a number of tomato plant pathogens, including *Phytophthora parasitica* (buckeye rot), *Pseudomonas syringae* (bacterial speck) and the *tobacco mosaic virus* (TMV). The ascomycete pathogen *Leptosphaeria maculans* causes blackleg disease on canola and CO_2 enrichment (800 ppm) was shown to decrease disease severity versus ambient in a chamber study (Zou et al., 2019). Therefore, different pathosystems can respond in a variety of ways and produce contrasting outcomes (positive, negative or neutral) that are dependent on the individual and interactive effects of elevated CO_2 levels on the pathogen, host and environment. This study thus extends the current knowledge by including the maize-GLS pathosystem.

Limitations of this study include the chamber effect that occurred and produced different results across the two trials. The nature of chamber studies is that field conditions cannot be replicated entirely, so certain dynamic interactions are absent in the Convirons, and any findings should ideally be replicated in a FACE trial. It is also necessary to note that a susceptible inbred maize line (B73) was utilised for this study, but more resistant cultivars, such as those planted on commercial farms, would likely not respond the same. The exact timing of gas exchange measurements can influence the outcome, which may have contributed to certain results not being reproduced in the repeat trial. Nevertheless, these effects should impact samples from the respective trials in a similar manner and should not prevent comparisons between treatments on a relative basis.

2.5 Future work

Significantly more research is required on plant-pathogen interactions at elevated [CO_2] to evaluate the range of effects on maize diseases, such as GLS. Long-term disease trials over multiple infection cycles (>10) are necessary to account for host/pathogen adaptation and particularly microbial evolution (Coakley et al., 1999, Chakraborty and Datta, 2003). Both FACE and enclosed chamber studies should be performed, as open field trials encompass the complete range of natural environmental conditions and chamber experiments can be used to analyse specific factors (single stressors) in a controlled environment. Investigating GLS disease under multiple biotic (pest diversity) and abiotic stresses (drought, heat, salinity and other greenhouse gases, e.g. ozone) is essential to elucidating the interactive effects of climate change. A broad range of CO_2 concentrations (400-1000 ppm), accounting for near and far future predictions, need to be tested to determine any potential threshold effects on GLS. Elevated CO_2 disease trials should examine a range of maize cultivars (particularly resistant varieties) and *Cercospora* species (across a wide range of inoculum concentrations) to determine any possible effects on maize defence factors (e.g. known resistance genes or QTL, SA, LOXs, JA, phytoalexins, phenolic compounds and immune receptors). Additionally, disease trials and physiological assessments (e.g. growth and development, gas exchange measurements, as well as any qualitative or quantitative assays) should be conducted under conditions that favour C_4 photosynthesis, including elevated light and temperature, since these are more analogous to natural settings. Examining *in vitro* fungal growth rate, colony morphology and secondary metabolite production (e.g. cercosporin levels in *Cercospora* spp.), avirulence genes, as well as fungicide activity, at elevated CO_2 levels is also required to understand pathogen development in the future climate.

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Appendix

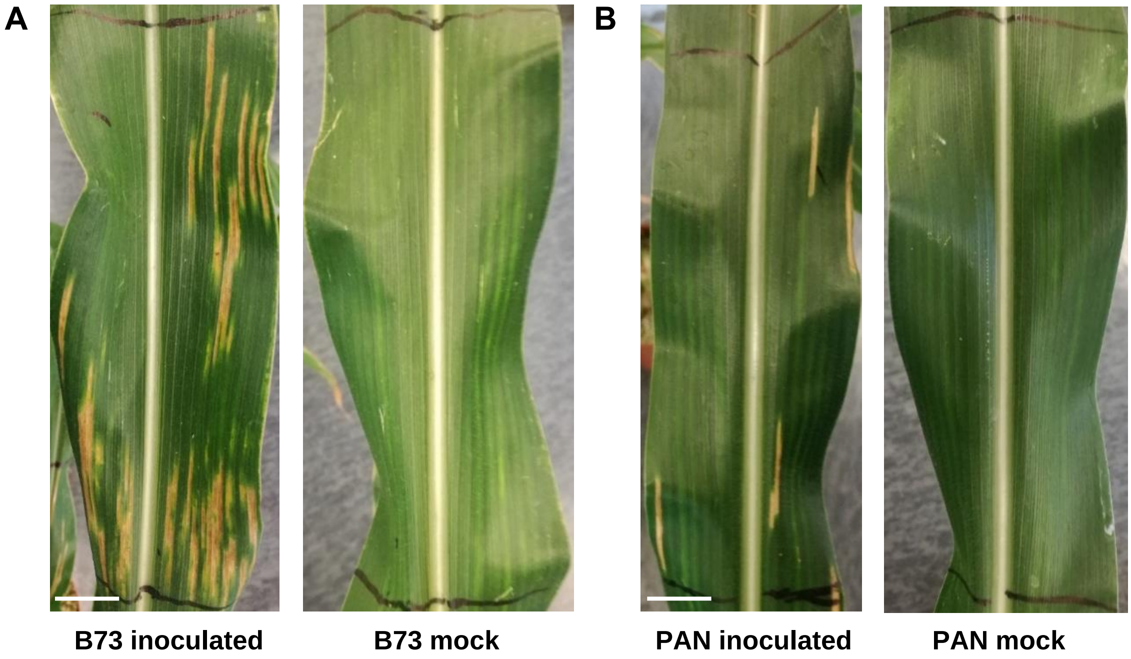

Figure S1. Representative photographs of GLS symptom development on B73 (A) and PAN 5A-154 (B) maize leaves at 415 ppm CO_2 at 42 dpi for the pilot study. Mock-inoculated leaves of both varieties remained symptomless throughout. Scale bar = 2 cm.

Table S1. Experimental trial design showing the inoculation parameters for the different CO_2 treatments.

Trial	[CO_2] ppm	[Inoculum] (conidia/ml)	V stage at inoculation	Inoculated plants	Mock-inoculated controls
1	415	7.8×10^5	V6	20	18
	700	7.8×10^5	V6	20	19
2	415	8.8×10^5	V6	18	17
	700	8.8×10^5	V6	18	17

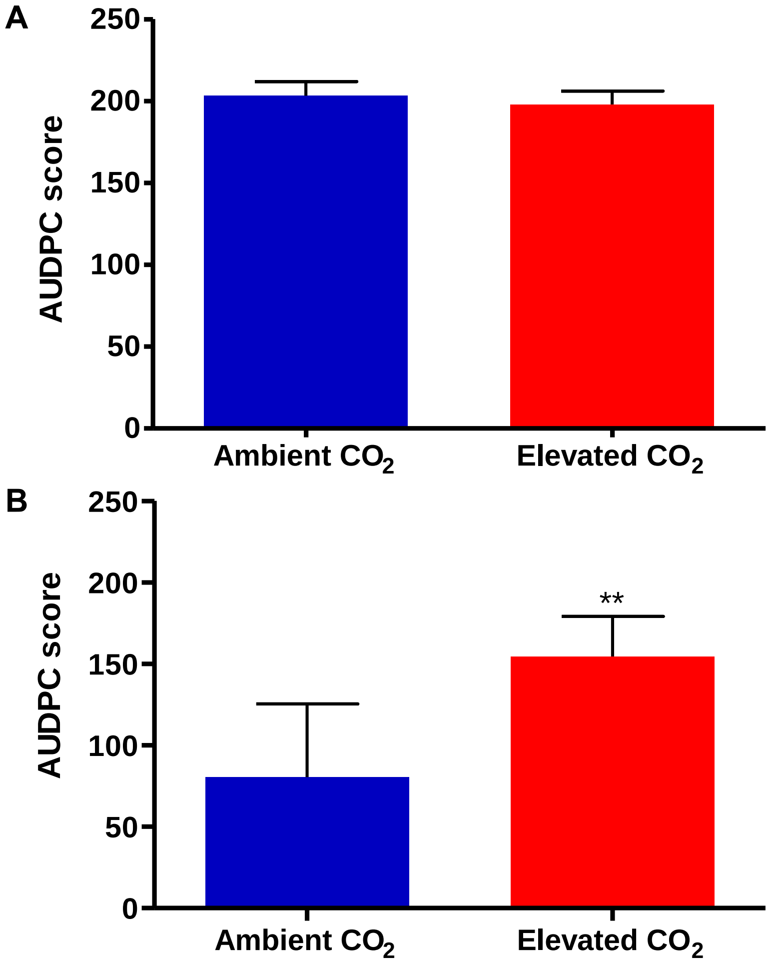

Figure S2. AUDPC scores for maize samples at ambient vs elevated [CO_2]. No significant difference in disease development (n = 10, ±SEM) was observed between CO_2 treatments for trial 1 (A), whereas a significant difference was reported for trial 2 (**$P<0.01$) (B), based on a Mann-Whitney *U* test (alpha = 0.05).

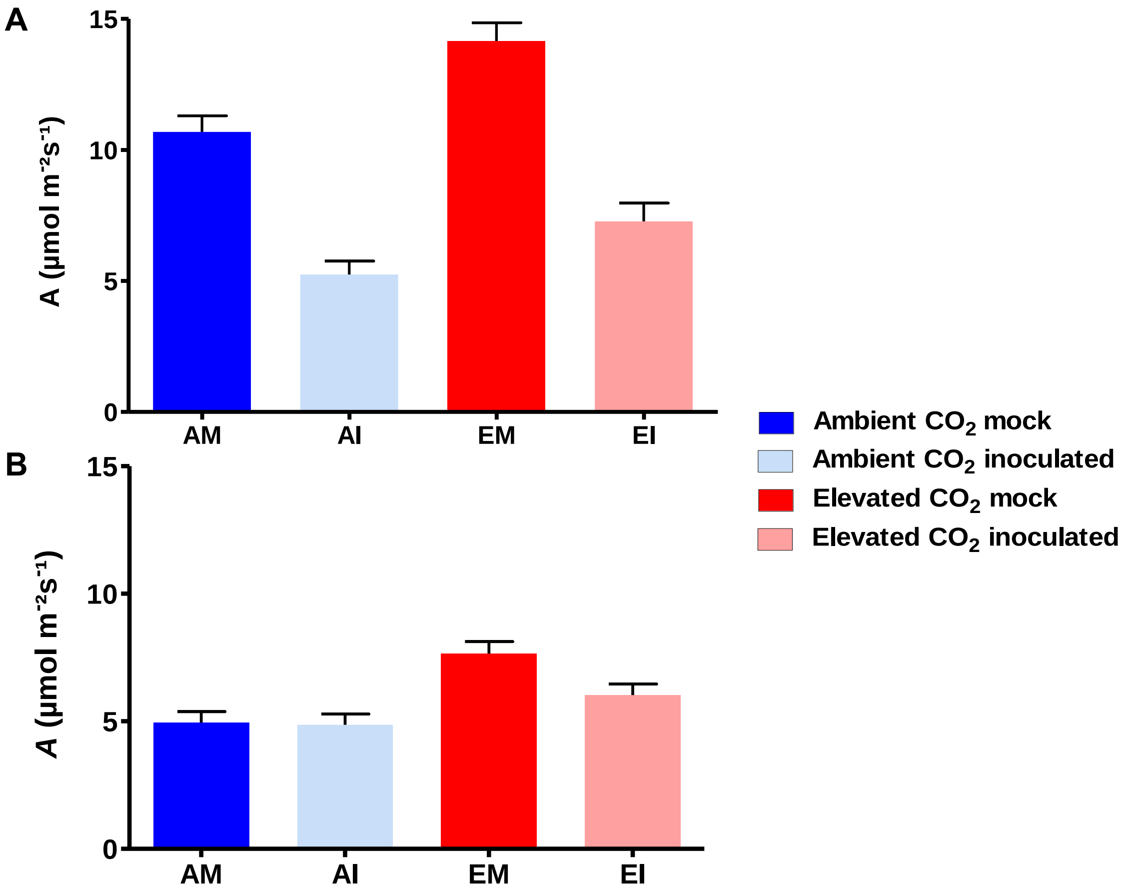

Figure S3. Net CO_2 assimilation rate (*A*) of maize leaves for the CO_2 and inoculation treatments. No significant difference in the photosynthetic rate (*n* = 48, ±SEM) was reported between the four treatments for both trial 1 (A) and trial 2 (B), based on a 2-way ANOVA with Bonferroni multiple comparison test (alpha = 0.05). Average values across 21, 28, 33 and 41 dpi samples are shown.

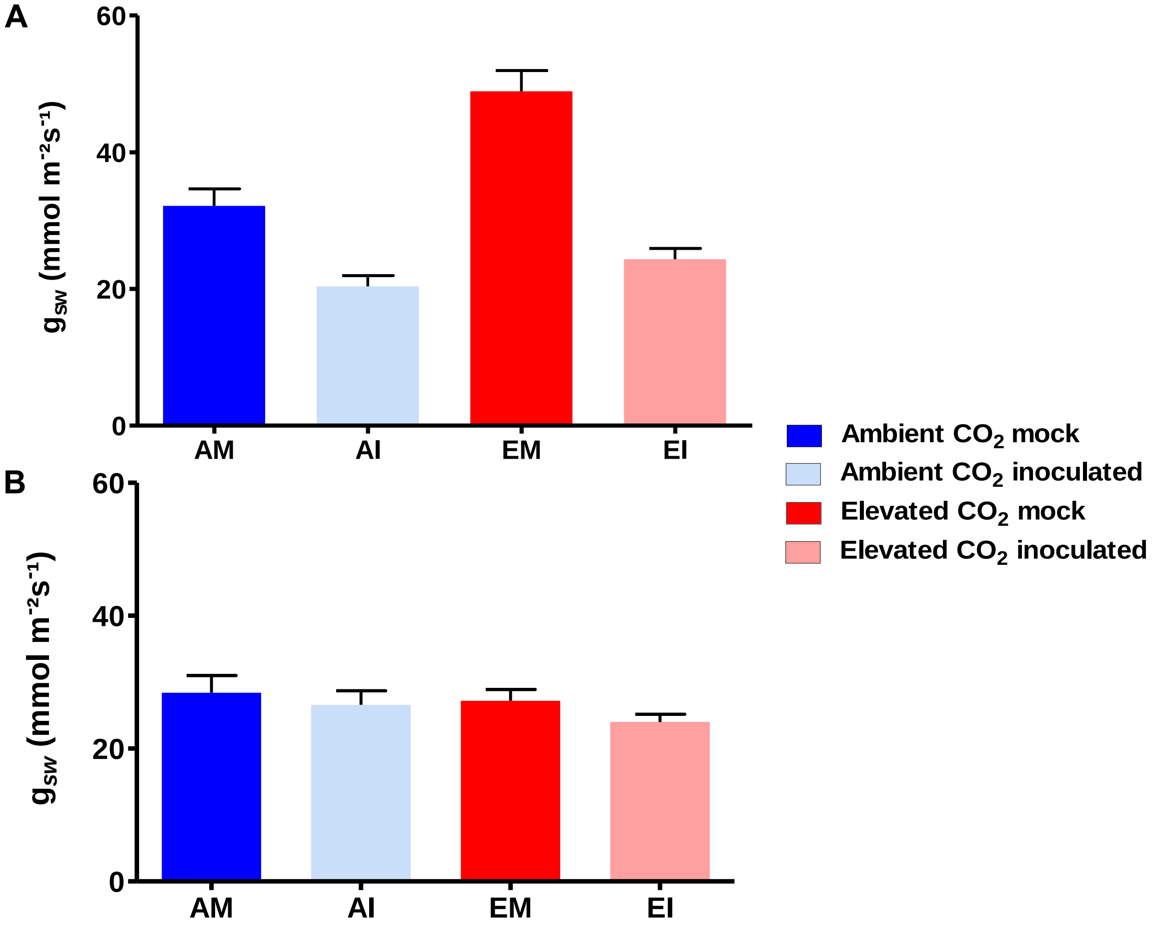

Figure S4. Stomatal conductance (g_{sw}) of maize leaves for the CO_2 and inoculation treatments. No significant difference in the stomatal conductance (*n* = 48, ±SEM) was reported between the four treatments for both trial 1 (A) and trial 2 (B), based on a 2-way ANOVA with Bonferroni multiple comparison test (alpha = 0.05). Average values across 21, 28, 33 and 41 dpi samples are shown.

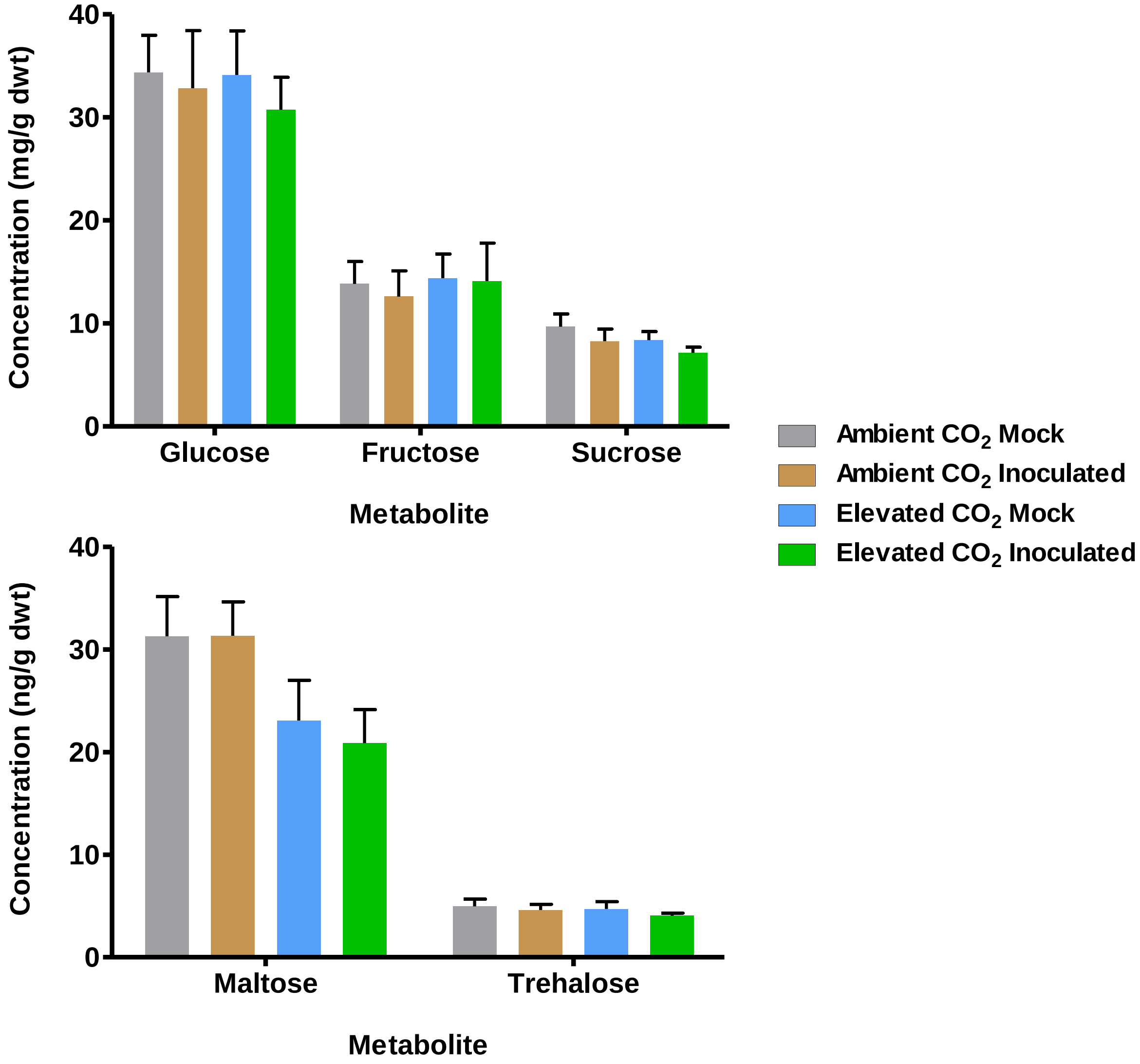

Figure S5. Maize leaf glucose, fructose, sucrose (mg/g dwt), maltose and trehalose (ng/g dwt) concentrations for the CO_2 and inoculation treatments. No significant difference in metabolite concentrations (*n* = 20, ±SEM) were observed between the four treatments for all sugars, according to a 2-way ANOVA with Bonferroni's multiple comparison test (alpha = 0.05). Dwt, dry weight.